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The Effect on Stem Cell Proteins through Laser Surface Treatment of Biomaterials

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The Effect of Stem Cell Proteins through Laser Surface Treatment of Biomaterials

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List of Abbreviations

- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)
- 3D Profilometry (3DP)
- Atomic force microscopy (AFM)
- Biotinylated detection antibody (BDA)
- Bone implant contact ratio (BIC)
- Bone marrow derived stem cells (BMC's)
- Bone morphogenic protein 7 (BMP-7)
- Calcium phosphate (CaP)
- Calcium phosphate cements (CPC)
- CPC/Bioglass composites (CPC/BG)
- Cationized gelatine (CG)
- Cetyl trimethylammonium bromide (CTAB)
- Chemically polished titanium (PT)
- Deproteinized bovine bone (DBB)
- Dimethyl Sulfoxide (DMSO)
- Dulbecco's Modified Eagle Media (DMEM)
- Erbium-doped: yttrium, aluminium, and garnet (Er: YAG)
- Extra cellular matrix (ECM)
- Foetal bovine serum (FBS)

- Food and Drugs Administration (FDA)
- Grit blasted and acid etched titanium (SLA)
- Human embryonic stem cells (hESC's)
- Human foetal osteoblast (hFOB)
- Human mesenchymal stem cells (hMSC)
- Hyaluronic acid (HA)
- Hydrofluoric acid (HF)
- Hydroxyapatite (HAP)
- Integrin alpha (5) beta (1) ($\alpha 5 \beta 1$)
- Intervertebral discs (IVD)
- Laser modified (LMT)
- Ligament Advances Reinforcement System (LARS)
- Lysine-triisocyanate-based polyurethane (PUR)
- Machined titanium (MT)
- Mesenchymal stem cells (MSCs)
- Micro-computed tomography (μ CT)
- Nano scale HAP/polycaprolactone (HAP/PCL)
- Nano-fluorohydroxyapatite (nFHA)
- Nickel-free high nitrogen stainless steel (NHS)
- Nickel titanium (NiTi)
- Osteogenic protein-1 (OP-1)
- Octamer-binding transcription factor 4 (Oct-4)
- Platelet rich plasma (PRP)
- Phosphate-buffered saline (PBS)
- Poly butylene succinate(PBSu)
- Poly ethylene glycol (PEG)

- Polyethylene terephthalate (PET)
- Poly ether-ether-ketone (PEEK)
- Poly vinyl alcohol (PVA)
- Plasma sprayed titanium with irregular projections (TPS)
- Scanning Electron Microscope (SEM)
- Secondary imaging (SI)
- β -tricalcium phosphate (TCP)
- Titanium base metal (BM)
- Titanium heat treated zone (HAZ)
- Titanium weldment zone (WZ)
- White Light Interferometry (WLI)
- X-ray photoelectron spectroscopy (XPS)
- Zoledronic acid (ZOL)

Abstract

The objectives of this project were to assess the effect of laser surface modification on the biocompatibility of polymeric biomaterials when used as a substrate for stem cell culture. Human mesenchymal stem cells were cultured on a pair of polymeric material substrates with varied incubation times and surface parameters. Poly ether ether ketone was selected due to its chemical and physical robustness as well as its use in load bearing implants as well as the potential to improve its efficacy via improved osteogenicity. The second material was Nylon 6'6 a common member of the Nylon family that is regularly used in a number of medical instruments and has been shown in literature to have potential in tissue engineering applications. Samples of both materials were cleaned and prepared for processing. The wattage of laser power used, the traverse speed, the pattern and the spacing between lines of the pattern were modify the material surface were all varied to produce differing surface patterns. A number of the surface characteristics of the processed samples were assessed in order to evaluate the effect of the individual processing parameters modified. These included:

- Material Wettability
- Surface Roughness
- Surface Chemistry
- Surface topography via SEM

Another objective of this project was to assess the effect of laser modified material surfaces on the growth rate of hMSCs. Alongside this, a proteomic assay as performed to assess if any differentiatonal bias enforced upon hMSC's by materials that have undergone processing. The was assessed via the expression of Bone

Morphogenic Protein 7 (BMP-7). Alongside this, the effect of laser surface modification was assessed using a cytotoxicity assay to assess cell growth rates using an incubation time course. The results of this study showed that PEEK was found to be the more hydrophobic material post processing though lower levels of laser energy per mm² squared were found to be more hydrophobic than samples that received higher amounts of laser energy. Nylon 6'6 samples on the other hand was much more hydrophilic in comparison to PEEK. Surface roughness was varied in both material, though the highest level of roughness was seen in PEEK as opposed to Nylon 6'6. That being said, PEEK generally was more difficult to produce a pattern on, this resulted in the some of the rougher surfaces as the surface simply vaporised at higher laser energy per mm². Both materials that underwent surface processing had totally different responses. When laser energy was applied to Nylon 6'6 it resulted in liquefaction which allowed for greater infiltration of atmospheric gasses. This resulted in an increase in an improved ration of nitrogen to carbon. PEEK on the other hand scorched as opposed to liquefying which resulted in a large increase in the level of carbon present. Cell response to modified surfaces was assessed using stem cell incubation followed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay to assess growth rates of hMSCs. Cells were incubated at 24 hours, 48 hours and 4 days to assess the effect over time. A statistically significant difference was found between the 24 hour and 48 hours and the 24 hours and 4 days. Cell growth rates were higher on samples that had undergone modification as opposed to unmodified control samples. Finally, the proteomic assay ELISA assay performed showed that there was no statistically significant difference between unmodified and modified material samples.

1.0 Introduction

1.1. Project Back Ground

1.1.1 Project Novelty

A number of elements of this project contribute towards its novelty while also having a grounding in a number of established research fields. A number of materials are currently used in both industry and research as what is known as the gold standard. A good example of this is Titanium and titanium based materials which are relatively chemically inert as well as physically robust. As a result of this as well as the lack of development required when compared to some materials, the majority of work in literature is based upon titanium and other first generation biomaterials e.g. titanium and steel alloys. Alternatively, polymeric materials offer an alternative that could be relatively cheaper, easier to process and more bioactive and one that offers more options for future study. As a Poly ether ether ketone (PEEK) was selected for a number of reasons. PEEK has been shown in literature to be very robust both physically and chemically as well as having a high temperature resistance. PEEK is often used in a number of industries ranging from the panels in the aerospace industry through to filters for biodiesel fuel. PEEK has also been highlighted in literature as a potential implant material for load bearing implants due to its aforementioned robustness as well as biocompatibility. Nylon is a family of semicrystalline thermo plastics that are relatively low cost have a number of uses in industry. Nylon 6'6 is one of the most common of these, made from two 6 carbon monomers it has been used in the production of items ranging from airbags and parachutes to swimwear and machine parts. Nylon 6'6 has also been used a number of times for a number of biological applications such as vascular grafts and sutures.

Laser modification offers a form of material modification that effects multiple surface properties in an area local to the point of modification while leaving other surface areas unmodified. This allows for the leading of cell growth as well as providing a high level of accuracy and relatability when compared to other methods of surface modification that are not able to effect specific areas without secondary processing. Aside from this, hMSCs are key focus of research due their differentiative capacity as well as their ease of isolation and expansion. As well as their potential in tissue engineering applications and prosthesis.

1.1.2 Project Value

The value from the project stems from the novelty of this form of project within the research area that is relatively undefined in terms of the materials being utilised within this project and the method of modification being used. While the literature that does exist is positive, this particular iteration i.e. polymeric biomaterials and laser surface processing. Both materials used have a pedigree for use within industry as well as literature indicating their efficacy in bio implant fields. PEEK has been shown in literature to have potential in load bearing implants and as an alternative material to titanium. Work in literature already shows that modifying the surface of can have a positive effect on bioactivity and biocompatibility. As a result of this, work of this type has direct application to current research paradigms and as well as materials that are already established in industry and literature. Laser surface processing has already been established in literature as able to modify a number of different properties including surface chemistry and topography to improve biocompatibility and bioactivity.

1.1.3 Project Objectives

The aims of this project were twofold, the goal of the initial work was to assess the benefit of laser surface treatment upon materials that have a defined surface profile in terms of both chemistry and cell response. This was assessed using surface topography, surface chemistry, surface wettability and using scanning electron microscopy. The second aspect of this project was to assess the growth rate of human mesenchymal stem cells on laser modified polymeric biomaterials as well as assessing if there was any form of differentiatinal bias towards osteogenic differentiation without the presence of differentiation leading media. This was measured using a proteomic assay to assess the expression of bone morphogenic protein 7 (BMP-7).

1.1.3.1 Null hypothesis (H^0)

Part One

The growth of hMSCs is not significantly different between PEEK and Nylon 6'6 after laser treatment.

- i. Between 24 hours and 48 hours
- ii. Between 24 hours and four days
- iii. Between 48 hours and four days

Part Two

The growth of hMSCs is not significantly affected by the incubation time the cells are subjected to;

- iv. Between 24 hours and 48 hours
- v. Between 24 hours and four days

- vi. Between 48 hours and four days

Part Three

The growth of hMSCs not significantly affected by the application of laser treatment on polymeric surfaces

Part Four

The amount of BMP-7 expressed by hMSCs is NOT significantly affected by the cell growth on laser treated biomaterials

1.1.3.2 Hypothesis (H¹)

Part One

The growth of hMSCs is significantly different between PEEK and Nylon 6'6 after laser treatment.

Part Two

The growth of hMSCs is significantly affected by the incubation time the cells are subjected to;

- i. Between 24 hours and 48 hours
- ii. Between 24 hours and four days
- iii. Between 48 hours and four days

Part Three

The growth of hMSCs is significantly affected by the application of laser treatment on polymeric surfaces

Part Four

The amount of BMP-7 expressed by hMSCs is significantly affected by the cell growth on laser treated biomaterials

1.2 Literature Review

1.2.1 Stem cells

Stem cells are defined in literature as the undifferentiated cells of a multicellular organism that give rise to a myriad of cells of different genetic lineages, as well as maintaining a set reservoir of themselves. Undifferentiated stem cells have a varying level of differentiative capacity, this is known as potency. There are a number of different levels of potency, the most encompassing of which is known as totipotency. Totipotency is the term applied to cells which can differentiate into effectively any differentiative lineage, a good example of which is newly fertilised embryonic stem cells. This is followed by pluripotency which describes a cell that can differentiate into the cells that comprise any of the three different germ layers. Within pluripotency exists a spectrum of differentiative capacity. Multipotent cells have the differentiative capacity to differentiate into the cells of a specific germ line (Jung, et al., 2011). For example, mesenchymal stem cells are able to produce bone, muscle, adipose and cartilaginous tissues. Oligopotency is used to describe stem cells that can differentiate to all the cells within a specific lineage, a good example of which is myeloid or lymphoid precursor cells. Finally, unipotency is used to describe stem cells that can only differentiate into a single cell lineage. This is generally used to describe specific precursor cells (Marieb & Hoehn, 2010).

Stem cells are often touted as having great potential in the future treatment of a plethora of conditions. They have also been proven to be a keystone in both the process of direct and indirect healing of tissue damage (Okana, 2014). A good example of which is work Doppler et al 2013 which discusses the role of stem cell therapies in future treatment methods in this case, specifically aimed at eliciting regeneration of damage in cardiac tissues. The work by Doppler et al 2013 describes the utilisation of bone marrow derived stem cells (BMC's) and adult stem cells from

adipose and cardiac tissue in the regeneration process. While the results tended to be poor in terms of clinical efficacy and outcome, from a conceptual point of view it means that stem cells have the potential to be utilised in the treatment of adult tissue injury. It also posits other potential avenues of treatment in the future, including the use of both novel and progenitor stem cells in new treatment modalities, as well as highlighting other tissue engineering techniques such as cellular reprogramming (Doppler, et al., 2013).

A number of different factors are considered when considering the suitability of stem cell donors, chief amongst these is age, the ability of hMSC populations to differentiate and proliferate decreases with the age of the donor, as well as the number of cell passages (Herman, et al., 2010). Therefore, embryonic cells and cells from younger donors with lower passage numbers are preferable to older cells and ones of a higher passage number, where cells are closer to senescence and more likely to have drifted genetically. Work by Xie et al 2011 corroborates this by stating that long term culture of human embryonic stem cells can also result in reduced differentiation potential and an increase in other associated errors such as genetic drift and morphological changes. In regards to stem cell culture, it is common practice to attempt to keep human stem cell cultures low in terms of passage number to minimise the effects such as genetic drift.

Work by Akhavan et al 2012 illustrates a key use of hMSC's in the investigation of the cytotoxic effects of graphene nano-platelets. It indicates that at most concentrations nano-platelets can induce cytotoxic effects through DNA damage and chromosomal aberrance, as well as through damage to the cellular membrane. The study concluded that the lateral size of the sheets is a key factor in the severity of their effect on cellular function. (Akhavan, et al., 2012) This article illustrates a key use of stem cell and tissue

culture in medical research. It indicates their usefulness when modelling the effect of chemicals and toxins at a cellular level, which is one of the first steps in clinical trials. An area of stem cell research which has received a great deal of interest has been the use of stem cells in the replacement of failed and critically damaged tissues. Not only does this issue present a tantalising opportunity to remove a number of serious medical issues, especially in regards to prosthesis and transplantation, this issue will only become more prevalent as the median age of the global population increase. Meaning the need for replacement organs and tissues will only rise, putting a greater strain on an already very limited, slow to replenish resource. For example, in conditions such as renal dysfunction, depending on the severity, transplantation can be the only curative treatment. While treatments such as dialysis allow treat the symptoms of the condition they do not treat the underlying cause. Stem cell based therapies and tissue engineering are one avenue through which the number of available organs could be increased as well as making the process of implanting and maintaining a new organ much simpler and more cost effective.

In recent years, a great deal of research has been devoted to controlling and differentiating cell lineages. A small number of examples of preclinical human trials have been published in literature, one of which is work by Berg et al 2014. In which a three-dimensional scaffold was derived from a donated trachea which was then implanted into a 76-year-old patient with tracheal stenosis. While unfortunately the patient eventually died of cardiac arrest, the post mortem histopathological investigation showed evidence of neovascularization within the scaffold and Alpha-actin 2 positive muscles cells. This indicates that the body not only accepted the larynx scaffold, but was also beginning to integrate with it. At the time of death, it was still providing the patient with an open air way for the patient, highlighting its efficacy for

its intended purpose. This being said, local and systemic fungicides were required to treat fungal infection and maintain the scaffolds viability (Berg, et al., 2014). Work in this theme is currently some of the clearest evidence of the potential efficacy of stem cells in tissue engineering purposes. Success in murine analogues has also been documented, such as in work by Greggio et al 2013, demonstrated the success of manipulating the growth media. This allowed the creation simplified spherical structures comprised of murine pancreatic progenitor cells. Complex organoids were also created, which were shown to undergo spontaneous differentiation and pancreatic morphogenesis (Greggio, et al., 2013). This further substantiates themes highlighted in literature which discuss the use of specific cellular growth medium to influence cell fate.

1.2.1.1 Human Mesenchymal Stem Cells

Human mesenchymal stem cells (hMSCs) are non-haemopoietic stem cells that were first discovered by Alexander Freidenstein in 1970. That resides within the stromal fraction of a large number of adult tissues including bone marrow and subcutaneous adipose tissue (Aldahmash, et al., 2012). Both human and non-human hMSCs are defined by their ability to differentiate into osteoblasts, chondrocytes and adipocytes after expansion *in vitro*.

Mesenchymal stem cells have garnered interest in the scientific community due to the relative lack of information available regarding them, as well as their ease of procurement and wide differentiation capability (Phillips, et al., 2010). They have demonstrated exceptional potential for clinical applications in a number of different fields ranging from tissue engineering to the production of new testing models for drug trials and genetics research (Aldahmash, et al., 2012).

While the number of hMSCs naturally present in the body's stromal fractions is relatively low, samples are expanded *in-vitro* to produce large enough populations for experimentation. Current methodologies for growth and expansion of stem cells use a combination of basal media and foetal bovine serum (FBS).

Cell based therapies are not a novel concept and bone marrow transplants have been a key treatment of a number of various malignancies, such as various forms of leukaemia (Pecorino, 2012). Stem cell based therapies utilising MSCs are one of the more promising avenues within regenerative medicine. They are greatly aided by their ability to home in on the site of injury and initiate repair processes. Whether this is via differentiation to replenish lost local cells or through modulation of the immune response as well as other local repair mechanisms. The ability of stem cells to moderate what proteins and soluble factors are produced in response to the specific needs of injured cells within the local environment truly point to their utility in the realm of wound healing as well as their utility in treating organ damage and dysfunction (Ma, 2010).

A good example of this concept in action is the positive effect of stem cell introduction to spinal cord injuries. Spinal cord damage can have a severe impact on patients and medical professionals and organisations. The negative mental and socioeconomic effects of spinal cord injury are also heavily documented and in some cases suicide (Griffiths, et al., 2012). Therefore, it is clear that the reduction of injury severity and potential morbidity and an increased rate of healing is beneficial not only to the patient but to healthcare professionals and organisations. Not only this, but it is economically beneficial for a sufferer to be able to return to work (Krueger, et al., 2013). Ohtaki et al 2008 discusses the reduction of neuronal death in murine animal models through the introduction of MSCs, with the aim of reducing damage and neuronal death following

global ischemia. This is achieved via immunological and inflammatory modulation on both a topical and the genetic level not only within themselves but by inducing changes within the cells around them (Ohtaki, et al., 2008). It is clear that hMSCs and stem cells have a whole have a large number of applications within a number of fields including tissue engineering and wound healing which were highlighted in this section.

1.2.1.2 Current State of Stem Cell Research

The differentiation potential of totipotent and pluripotent stem cells and their expansion capability, have resulted in a great deal of scrutiny in literature. Currently, both embryonic and adult stem cells are the subject of a great deal of scientific interest from a wide range of fields. Work by Ikebe & Suzuki 2014 reported the current state of stem cell research for future treatment methods. The article indicates that current trials generally produce feasible results that highlight the benefit of stem cell therapies in future treatment methods. However, it also states that some ambiguity exists between different studies. This is put down to the variability between different methods of isolation and expansion of mesenchymal stem cells MSCs from donors. The article then suggests that the development of an international standard for isolation and expansion would be beneficial and also allows for the removal, or at the very least minimisation of a potential source of error and variance between studies (Ikebe & Suzuki, 2014).

One of the key potential uses of stem cell based therapies hypothesised in research, is replication and replacement of failed and damaged tissues. The current strain upon the pool of available donor organs will only become more severe as the number and median age of the global population increase. This means that the need for replacement organs and tissues will also rise. This will put a greater drain on an already limited resource. While also being one that lacks the ability increase in parity

and could in fact become more scarce, due to the increased prevalence of conditions such as type II diabetes, which can impact donor viability in some organs (Rithalia, et al., 2009). With some forms of organ dysfunction such as renal failure, transplantation is one potential avenue of treatment and in severe and end stage cases is the only option that is curative. Stem cell based therapies and tissue engineering are one potential avenue through which the number of available organs could be increased as well as making the processes of implantation and maintenance of a new organ relatively straightforward (Vacanti, 2012).

Long term rejection and graft versus host disease are key considerations when considering candidates to receive a donor organ. It can also become an issue especially over long periods of time, depending on the HLA match of the donated organ with the host (Hall & Yates, 2012). At the end of 2012, roughly 8,000 people in the UK and almost 77,000 people in the US are currently waiting for some kind of organ or tissue transplantation. Approximately one thousand people who are waiting for a transplant in the UK die while waiting for a compatible organ to become available (Wright & Barlow, 2014).

Weiss 2014 reported the current status of stem cell and regenerative therapy in lung biology and disease. The article discusses how cell based regenerative therapies are being investigated as a potential possibility of addressing the current severe need for new therapeutic treatment methods of lung dysfunction (Weiss, 2014). A number of studies are currently ongoing that are investigating the potential of *ex-vivo* bioengineering. This includes one that details the potential of this process in lung bioengineering, both for therapeutic and research purposes. The use of both natural and artificial scaffolds offers the potential of creating an artificial organ or tissue by seeding stem cells onto a scaffold. Both natural and artificial scaffold have been used

in previous work as in work by Berg et al 2014. In which it is highlighted that cells grown upon a scaffold can even regain functions that they no longer possessed (Berg, et al., 2014). Work by Wagner et al 2014 describes how pre-formed natural scaffolds can be prepared from human organs from cadavers via the decellularisation. These decellularised connective tissue matrices can then be seeded with stem cells harvested from a patient. In this manner it is possible to create an allogeneic organ directly from a patient's own cells. (Wagner, et al., 2013). Nonetheless, in specific regard to the decellularization of lungs scaffolds, no optimal approach is currently agreed upon in literature. This does not mean that success is unmanageable. As in other cases such as in work by Berg et al 2014, decellularised scaffold implantation and seeding with the patient's own stem cells was carried out with some functional success (Berg, et al., 2014). However, the structure of a lung is much more complex than that of the trachea, so how well the methodology will scale is a different matter entirely. Currently, the oesophagus, the trachea and skeletal muscle structure have all been repaired and reproduced in human and animal models using this methodology (Badylak, et al., 2012).

Work by Tapias and Tot 2014 illustrates the current state of research into the use of scaffolds in decellularised scaffolds produced from donated organs in an attempt to produce new, functioning organs. It indicates that while pluripotent stem cells show the potential to be used to reseed the scaffolds as was also discussed in work by Berg et al 2014 and work by Wagner et al 2014. The article highlights the difficulty of leading cell differentiation within a scaffold to produce a viable organ. This means that the desired cell type cannot be specifically elicited (Tapias & Ott, 2014). However, the use of biomaterial surface modification gives the potential of leading cells down a specific

lineage via topographical and chemical changes or possibly immersing a seeded scaffold in specific differentiation media and growth factors (Wang, et al., 2011)

That being said, a great deal more research and ground-breaking must be before bioengineered organs can become anything more than a theoretical exercise. Scaling to human size may also be troublesome, however much less so than leading differentiation. This need can currently be met *in vitro* via the use of specific media to lead cells down a specific lineage and the mechanisms that are utilised in this instance could potentially be applied to either *in situ* organ development or *in vitro* bioengineering for implantation (Wagner, et al., 2013).

1.2.2 Bone Morphogenic Protein-7 and Osteoblast Differentiation

Bone morphogenic protein-7 (BMP-7) or Osteogenic protein-1 (OP-1) as it is also known, is a multi-functional growth factor that is member of the BMP family of secreted signalling molecules. BMP-7 have been shown to induce ectopic bone growth, and are members of the TGF- β superfamily of proteins. This superfamily has been shown to play a crucial role in the differentiation of hMSCs into both osteoblasts and chondrocytes (Chen, et al., 2002). BMP-7 is involved in a plethora of different roles within the body ranging from osteogenic differentiation among mesenchymal stem cells, to the promotion of Langerhans cell differentiation in both murine and human models (Yasmin, et al., 2013).

A number of studies have focused specifically on the signal transduction pathways utilised by members of the BMP family and chiefly, BMP-7. The literature shows that the activation of BMP receptors initiates downstream phosphorylation of receptor-regulated SMADs. This results in signal transduction followed by the formation of hetero-oligomeric complexes, which are comprised of common mediator SMADs and receptor regulated SMADs. These complexes then translocate into the nucleus of cells

where they regulate the transcription of downstream target genes. Down regulation is carried out by inhibitory SMADs as well as extracellular antagonists, such as noggin, to inhibit BMP receptor binding (ten Dijke, 2006). A good portrayal of the mechanisms of action of SMADs can be found in work by Cao & Chen 2005, which describes Smad 1, 5 and 8, which are direct downstream molecules of BMP family receptors. All of which are crucially involved in the process of BMP signal transduction in humans (Cao & Chen, 2005).

Additional studies performed in human and animal models with naturally occurring BMP mutations illustrate the importance of BMP signalling to a number of vital processes within the body and during foetal development. These include studies into cartilage and heart development and as well as postnatal bone formation. Work by Segklia et al 2012 discusses neural development in mice and the effect of non-specific BMP-7 knockout therein. The article describes how the knockout of BMP-7 resulted in impaired neurogenic activity, as well as reduced cortical thickening and a reduced level of attachment between the glia and the meninges. As well as this, there was a significant reduction in the action and lifespan of neural progenitor cells (Segklia, et al., 2012).

One of the most reported, and thus far effective, uses of BMP-7 reported in literature relates to its application in the healing of bone non-unions following severe trauma. In 2001, rhBMP-7 was approved by the Food and Drugs Administration (FDA) as an alternative to the use of autographic bone insertion in long term non-unions (Ong, et al., 2010). A three-year multicentre study carried out by Kanakaris et al 2008 clearly demonstrated its effectiveness in this particular treatment modality. Prior to the application of BMP-7 based treatment methods, the median healing time of tibial non-unions reported, was approximately 23 months. Reported healing times within the

review ranged from roughly 9 months in some minor cases to around 317 months, over 26 years in the worst cases. The results of the study showed that of the 68 patients treated, 89.7% demonstrated verified non-union healing. An average healing time of approximately 6.5 months was reported with the range of healing times being from three to fifteen months. Even the maximal recorded healing time was over 23 months shorter than the maximal healing time recorded not in conjunction with the use of BMP-7. It is acknowledged within the study however, that other factors may have compounded to hinder that particular subject's recovery time. It is also of note that no adverse reactions were documented at any stage of the review with BMP-7 use (Kanakaris, et al., 2008).

This study illustrates the benefits of BMP-7 in human models for fracture healing and is compounded by work by Giannoudis et al 2009, who also participated in the 2008 study by Kanakaris. The study described how up 10% of the 6,200,000 fractures that occur annually in the United States develop either some form of delayed union or medically verifiable non-union (Tzioupis & Giannoudis, 2007). Work by Giannoudis et al 2009 describes how BMP-7 stimulates the differentiation and proliferation of pluripotent hMSCs as well as angiogenesis via vascular endothelial growth factor derived from osteoblast cells, which is beneficial to the healing of non-unions as well as osteoinductive bone growth. In this study, data on 45 patients suffering from atrophic aseptic non-union of long bone fractures was obtained from two trauma centres between 2003 and 2006. All patients were treated with BMP-7 and an autologous bone graft. The results showed that one hundred percent of non-unions healed as assessed by both clinical and radiological assessments. Average healing time was 5.5 months with a range of three to fourteen months by clinical assessment and 6.8 months via radiological assessment with a range of four to sixteen months.

The efficacy of this treatment avenue and of BMP-7 in this form of injury is demonstrated by the fact that 39 of the 45 patients returned to their previous occupation upon completion of treatment. All of the six who did not return to their previous occupations suffered from more severe non-unions with all being attributed to soft tissue contracture and joint stiffness. (Giannoudis, et al., 2009). The aforementioned studies highlight the use of BMP-7 as a method of non-union healing through the stimulation of the differentiation and action of osteoblast cells. However, the increase in action is nonspecific, BMP-7 also affects the differentiation rate of osteoclast cells, resulting in an increased level of bone reabsorption and remodelling. In short, osteoclastic action increases in line with osteoblastic action in a form of homeostatic balance (Pederson, et al., 2008). Bosemark et al 2013 investigated the process of bone healing using rat models, using a number of different methods of bone healing. Natural healing with an autograph, autograph healing augmented with BMP-7 and autography healing augmented by BMP-7 in the presence of an adjunct in the form of biphosphate in this case, zoledronate. The results of the article show how the callus volume was increased two fold in the presence of BMP-7 and four fold in the presence of BMP-7 and a systematic adjunct, in both instances producing a very statistically significant value. Mechanical strength tests of the callus were also measured, again the BMP-7 and BMP-7 with adjunct samples both outperformed the autograph only calluses by two and four folds with both values being statistically significant. (Bosemark, et al., 2013). Bosemark indicates that the level of osteoclastic action can be controlled potentially allowing for quicker healing times of bone trauma. However, the effect of increased callus volume upon surrounding tissues and long term effects is unknown and should be investigated.

The well organised albeit complex mechanism of bone healing usually allows for the healing of breaks in that it allows the bone to retain the same strength it had before it was broken. However, in some cases this does not occur correctly, and results in a longer term breakage in the bone structure, which becomes a non-union in 5% to 20% of cases (Bajada, et al., 2009). A number of different types of non-union have been reported, categorised by trophic nature and septic status. BMP-7 has shown to be useful in the treatment of atrophic non-unions as has been mentioned previously has a good record of improving patient outlook in comparison to non BMP-7 based modalities. Especially in atrophic non-unions where local and cellular osteogenic mechanisms have failed, this allows for the process to be restarted.

1.2.3 Biomaterials and Modification of Surface Properties

Controlling the rate at which cellular reactions occur has been shown to be of great importance in a number of different fields of research for a number of years (Murugan, et al., 2009). One of the most effective methods of doing this is via the manipulation of the microenvironment in which the cells are grown. From a biomaterial perspective this is accomplished via the manipulation of surface properties such as roughness and chemical composition.

One key consideration when designing an implant or selecting a biomaterial for use within the human body is how well the implant is able to withstand the forces that the tissue itself would be subjected to. Biomaterials and implants are designed to replace bone tissue are designed with the ability to withstand the same if not greater, mechanical and kinetic strain for a prolonged period of time. Nevertheless, regardless of its durability, for a biomaterial to be a viable implantation candidate a good quality bone-implant reaction is required (Castellani, et al., 2011). The quality of this reaction is dependent upon the surface chemistry and topography of the biomaterial and how

they interact with the bone tissue (Zreigat, et al., 2005). Biomaterials regularly used in implants tend to only partially satisfy these requirements, two examples of which are Titanium and poly ether-ether-ketone (PEEK).

PEEK is a semi-crystalline synthetic thermoplastic polymer comprised of ketone groups, benzene rings and ether groups that has become popular part of orthopaedic and spinal treatments (Ma & Tang, 2014). It is formed of repeating polymer units which generally comprises two benzene rings joined by an ether group and a ketone group on the other end as can be seen in figure one.

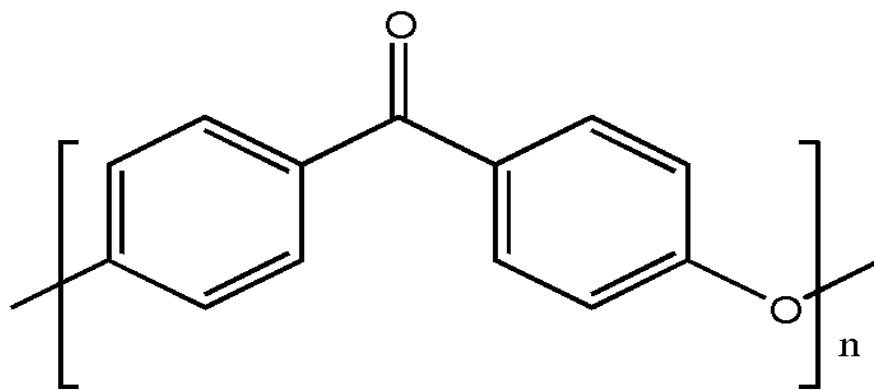


Figure 1: A repeating unit of PEEK containing two benzene rings joined by a Ketone group followed by an ether group (Wang, et al., 2012)

It is known for having physical properties including high wear resistance and low moisture absorption and has been utilised in implants due to its relative inertness. It is often considered as a substitute for metallic biomaterials due to its low elasticity modulus of approximately 3-4 gigapascals (GPa) (Akkan, et al., 2014). This means it is generally used as an alternative to titanium in load bearing implants (Kurtz & Devine, 2007). It can be described as a robust and inflexible material both physically and chemically.

Conversely, Nylon 6'6 is a specific polyamide molecule which belongs to the Nylon family which is utilised most regularly in the textile and plastics industries. It is known

to have a similar elasticity modulus as PEEK, around 3.3 GPA but have a much higher thermal conductivity and is generally more malleable. Nylon 6'6 is the result of a condensation reaction between two amide molecules resulting in the molecule shown in figure two. A molecule of water is also produced as a by-product.

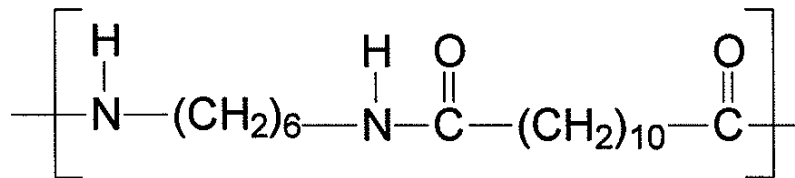


Figure 2: A repeating unit of Nylon 6'6 comprised of two amides via a condensation reaction yielding the repeating unit and a molecule of water (Frost, et al., 2010)

Both of these materials are known for their exceptional chemical resistance and their resistance to kinetic wear and tear but their substandard bioactivity generally as a result of their aforementioned chemical stability. This results in reduced cellular adhesion and often leads in poor bone-implant reactions (Niinomi, 2008). This leads to the formation of an intervening layer of fibrous tissue surrounding the point of contact.

One potential option to improve the efficacy of materials such as titanium and PEEK while still maintaining their bulk properties is the modification of their natural surface properties with the aim of enhancing particular properties or not others (Bartalena, et al., 2012)

1.2.3.1 Laser Surface Modification

One method through which surface modification can be performed is the application of laser light to the surface of a material. Laser based methods are an excellent candidate due to their ability to modify the surface properties of a material while being able to maintain the bulk properties of the material unaltered. Laser methods also possess a relatively minor operating cost in terms of materials and maintenance as

well as having a high resolution and operating speed (Riveiro, et al., 2013). A number of different methods of modifying surface properties have been pioneered and documented in literature, such as the coating of an initial material with bioactive materials such as calcium phosphate or bioglass (Naghib, et al., 2012) to the production of highly controlled macro and microstructures as a method of modifying a biomaterials surface topography.

Laser treatment of common biomaterials such as PEEK has garnered an increased level of interest in recent years and work by Riveiro et al 2013 discusses the laser surface modification of titanium and PEEK and methods through which it can be enhanced. In this paper the roughness and wettability of PEEK is modified using laser treatment of three different wavelengths of laser radiation ($\lambda = 1064, 532, \text{ and } 355 \text{ nm}$) with the intention of discerning the best processing conditions to enhance the roughness and wettability, thereby affecting the cell adhesion properties of implants. Results showed that the ultraviolet frequencies of laser radiation ($\lambda = 355 \text{ nm}$) is the most suitable one to enhance surface wettability of PEEK (Riveiro, et al., 2013).

While the ultraviolet wavelength laser light is reported in literature superior in terms of producing a pattern upon a biomaterial, as is noted in Riveiro et al 2013. Waugh et al 2008 also discusses the differences between different laser based systems which produce infrared wavelength light, in comparison to F_2 excimer laser systems which are based within the ultraviolet spectra (Riveiro, et al., 2013). The article indicates that the F_2 excimer based system was superior to the CO_2 based system used in this test, which corroborates the findings of Riverio et al 2013. This could be due to the use of direct material ablation method of material removal used in the excimer laser system as opposed to the bond vibration method used in CO_2 methods (Waugh, et al., 2008).

However, the article does mention that increased oxygen concentrations and increased polar molecule content are observed in the infrared based laser as opposed to the excimer laser system (Waugh, et al., 2008). This could indicate that CO₂ lasers may be more effective when attempting to modify material properties in regards to biocompatibility. However, excimer lasers are still preferable if topographical modification is required with a minimal amount of modification to chemical properties (Waugh, et al., 2008).

Work performed by Chan et al 2012, which investigated the effects of laser surface treatment upon nickel titanium (NiTi) alloy from both a topographical and a chemical perspective. Results showed that which different topographical characteristics did have an effect upon cell morphology, growth and orientation. Chemical composition was more beneficial to cell growth than topography (Chan, et al., 2013). However, this does not mean that topographical modification has nothing to add. Increased roughness characteristics result in increased cell adhesion and conversely smoother surfaces result in cell spreading (Biazar, et al., 2011).

In the case of titanium, the application of laser treatment can result in the formation microstructures with an increased cellular adhesion. This can be seen demonstrated in Faeda et al 2009 which discusses the removal torque required to remove laser modified (LMT) and machined (MT) titanium implants in rabbit tibias. The article states that across the entire experiment the LMT implants consistently had a higher removal torque than their MT implant counterparts. The LMT implants were also shown to have a much deeper and regular topography as well as an increased concentration of oxygen on their surface when compared to MT implants (Faeda, et al., 2009). The improved topography resulting in increased cellular adhesion due to the deeper grooves (LMT $R_a = 1.38\mu\text{m} \pm 0.23\mu\text{m}$) as opposed to the significantly shallower ones of

(MT = $0.33 \mu\text{m} \pm 0.06\mu\text{m}$) as well as the increased levels of oxygen available at the surface in LMT implants resulted in an increased level of healing and bone implant interaction resulting in higher torque forces required for removal. A clear difference between the roughness and topography between LMT and the MT implants was observed with a statistically significant result being produced (Faeda, et al., 2009).

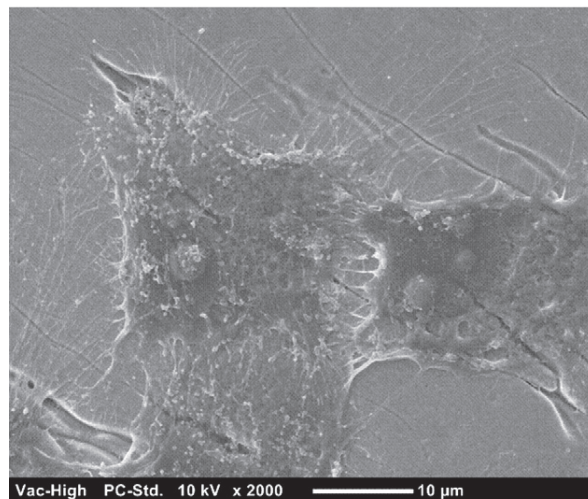
However, a study by Galli et al 2011 hints at caution, as it indicates that erbium-doped: yttrium, aluminium, and garnet (Er: YAG) laser with a 10Hz at both at 200mJ, could have a negative effect on cell proliferation and resulted in a lower production osteoprotegrin (Galli, et al., 2011). However, Ayobian-Markazi et al 2013 reported using Er: YAG at 100mJ indicates that there were no adverse effects related to laser treatment and in fact, there were positive variances when compared to the control group such as increase cell viability, producing a result that is shown to be statistically significant. The test group in this study also exhibited a statistically significant increase in surface wettability and roughness in both instances (Ayobian-Markazi, et al., 2013).

Work by Ayobian-Markazi et al 2014 compounds the previous work they performed in 2013 by highlighting the benefits of the modifications of biomaterial surface properties via laser treatment. A key result of this study was that while the cell viability rates of the two test groups showed little to no statistically significant result, the control groups exhibited a much lower viability rate which was found to be substantial with a very high degree of significance (Ayobian-Markazi, et al., 2014). This points to the significant potential effects of laser surface treatment on biological processes. One potential cause for the caution is hinted at by work by Galli et al 2011, which demonstrated that the over irradiation of the samples at 200mJ resulted in decreased cell growth. This could be the result of inferior surface chemistry due to radiation over exposure or topography, or potentially both (Galli, et al., 2011). This raises the question of which

is more important, surface chemistry or surface topography. Regardless of this question, these studies do justify the current levels of interest in laser treatment as it does modify cellular behaviour as a direct result of its use.

Chan et al 2013 validates this further by describing how the modified topography of the weldment zone allowed for increased cell adhesion due to the dendritic pattern that formed as a direct consequence of the rapid solidification of the titanium's crystalloid structure along the most energetically favourable crystallographic pathway for heat diffusion. This is demonstrated in figure 3 taken directly from the paper (Chan, et al., 2013).

(a) WZ (as-welded)



(b) Magnified SEM image of (a)

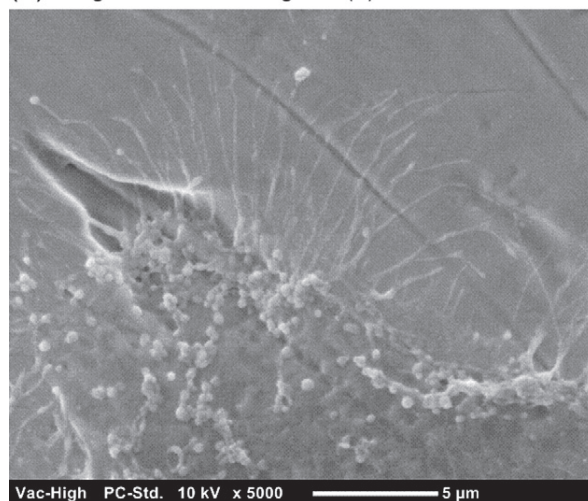


Figure 3: hMSCs conform to the topographical elements of their environment as can be seen in picture a and b. They use this to increase their surface area (Chan, et al., 2013)

Chan et al 2013 also describes how the laser treatment also allows for a more amicable surface chemical composition. Laser treatment results in higher titanium to nickel ratio as well as increased levels of oxygen at the surface. (Chan, et al., 2013). This is due to the formation of titanium oxide as a result of the rapid melting of titanium around the lasers path and combination with atmospheric oxygen as it cooled. The fact that this trend is repeated in the heat treated zone to a lesser degree. This increased oxygen content alongside a reduced concentration of nickel results in an increased cellular growth rate in the weldment zone and possibly in the heat treatment zone (Chan, et al., 2013).

Other metals used for implantation such as nickel such as is found in some steel alloys, generally has an adequate level biocompatibility for use in implantation. Nevertheless, it is reported that between ten and fifteen percent of the population has some form of hypersensitivity to nickel (Adala, et al., 2011). It has also been reported that this is increasing in prevalence, to the point where legislation has been proposed and enacted to reduce levels of human exposure (Schram, et al., 2010). More commonly used materials such as titanium generally do not elicit any kind of hypersensitivity response. However some more recent literature posits that reactions do occur and that the lack of positively identified cases is due to rarity and the lack of a standardised test as is the case with other substances (Vijayaraghavan, et al., 2012). As a result of a great deal of research has been performed into the effect of the modification of surface properties including surface treatments to improve biocompatibility as well as characteristics such as wear and corrosion resistance.

However, one of the key future trends in biomaterial research is to develop an osteogenic implant surface to allow for quicker healing and has many potential applications in both medical and dental procedures that involve the implantation of foreign materials into bone tissue (Pattaniaik, et al., 2012).

1.2.3.2 Polymeric Biomaterial Alternatives

Polymeric biomaterials have been highlighted in literature to have a beneficial effect on cell growth and proliferation (Dawson, et al., 2008). A number of materials have been highlighted in literature over a number of years to have potential in this regard.

Polyethylene terephthalate (PET) is one such material polymeric biomaterial which is a member of the polyester family of materials. Among its plethora of current uses, it has been recorded in literature that it has a beneficial effect on cell growth in a number of different ways. PET has been utilised as an implant material within the body for a number of years, a key example of which is the Ligament Advances Reinforcement System (LARS) which utilised PET to mimic ligamentous structure and enhance cell ingress into the ligament to increase anchoring (Mascarenhas & P.B, 2008). On top of this a large amount of research has been undertaken to further improve the biocompatibility of PET, some of which was aimed specifically at improving artificial ligament function.

Work carried out by Li et al 2012 investigated the effect of hyaluronic acid (HA) and cationized gelatine (CG) on hydrolysed PET films. The article showed that *in-vitro* HA-CG modified PET films exhibited enhanced cell growth and adhesion. While concurrently suppressing the expression of genes related to the inflammation process, something that is normally observed in pure PET grafts (Li, et al., 2012). The article goes on to discuss the efficacy of HA-CG PET ligaments in animal models, which is further explored in a follow up article by Cho et al 2013 which investigates the use of

HA-CG PET artificial ligaments in porcine models. The results showed that there was a significant difference in bone graft complex between the HA-CG PET and an unmodified PET control at both the distal femoral site and the distal tibial site generating a statistically significant result in both instances, other sites however did not generate any form of statistically significant data. The article concluded that CH surfaces on PET based artificial ligaments had a positive effect on artificial ligament osteointegration within the bone tunnel at the distal site (Cho, et al., 2013). This is all further summarised in a review article by Li and Chen 2014 which discusses previous work as well as summarising future potential research avenues. The review does state however, that the load to failure observed in PET based artificial ligaments is still inferior when compared to natural ACL ligaments, despite the increased osteointegration observed (Li & Chen, 2014). The literature indicates the benefit of chemical and topographical modification of PET surfaces in both *in-vivo* and *in-vitro* models. Further research into potentially superior coating materials and processing methods, as well as possibly more suitable polymeric alternatives to PET is one potential avenue of future research.

LARS ligaments are made of interlocking fibres that form a porous structure which has been shown to promote endothelial growth as well as promote cellular ingrowth (Lui, et al., 2006). The porous structure, when considered alongside the previously mentioned coatings, could result in an enhanced cellular response greater than the parts of either of them separately.

An article published by Zouani et al 2010, which discussed the grafting of mimetic protein markers to PET based biomaterial substrates with the aim of increasing pre-osteoblast adhesion. Peptide substrates matching residues 73-92, 89-117, and 68-87 of BMP-2, BMP-7 and BMP-9 respectively as well as adhesion peptides (GRGDSPC)

and were grafted to PET based substrates. The efficacy of which was evaluated in a number of different ways including transcription factors that are critical to osteogenic differentiation and cell morphology between induced cells grown on PET based materials and those that were grown normally. The results showed that the morphology displayed by cells grown upon these surfaces were different than that displayed by cells within a state of differentiation or ECM production (Zouani, et al., 2010). Induced cells also had an increased thickness than their non-induced counterparts, a trend that was repeated in the surrounding ECM that the cells produced. The article went on to demonstrate the efficacy of these mimetic peptide which tally well with the use seen in whole peptide chains as was noted in studies into the use of BMP-7 in the healing of tibial non-unions (Giannoudis, et al., 2009).

1.2.3.3 Antibacterial Properties in Biomaterials

Another area of research that is receiving a great deal of interest from within the scientific community is the use of material surface properties to enhance antibacterial properties of materials. An article previously mentioned, by Wang et al 2014 touches on this subject when it mentions how the materials formed were able to prevent biofilm formation and inhibit bacterial growth (Ivanova, et al., 2012). Antibacterial properties of material surfaces have been noted on a number of occasions in nature. An article by Ivanova et al 2012 discusses the how cicada was initially believed to possess anti fouling properties, preventing biofilm formation. However, the article discusses how when *P. arigenousa* are incubated on cicada wings, they are not repelled by super hydrophobic action as previously thought, but physically pierced by nanopillar arrays on the surface of the wings resulting in the death of the cells (Ivanova, et al., 2012).

The article discusses how cicada wings are an effective antibacterial agent preventing damage and degradation and introduces the concept of naturally occurring

antibacterial properties. An article previously mentioned by Wang et al also mentions this concept in that biocomposites with HAP have an increased antibacterial action in comparison to pure PEEK samples (Wang, et al., 2014). This is an area of research with a massive amount of ongoing research and also has application in a number of different fields from medical equipment such as catheters (Glinel, et al., 2012).

This area is one of interest with a great number of research opportunities within this form of research which also is relatively simple to test in laboratory conditions.

1.2.3.4 Ceramic Biomaterial Alternatives

There is a myriad of alternatives to most of the materials currently used in implantation. Some alternatives such as ceramic based implants have been the subject of intense research as is highlighted in a systematic review published by Andreiotelli et al in the Journal of Clinical Oral Implants Research. The reviewers search and subsequent screening found over one thousand individual publications, which were eventually reduced to 25 core studies. The studies indicated that the available data is not sufficient to recommend ceramic materials as an appropriate biomaterial in dental use at the current time. However, the study then went on to say that zirconia based implants do have potential, despite this, more data is still required (Andreotelli, et al., 2009). This systematic review gives a good sounding of what is currently available within the field, that being said the article specifically states that no clinical trials regarding the outcome of zirconia and alumina substrates could be found.

Since then, further investigation has shown that some of the properties Zirconia possesses lent it well to use as a component of dental implants. Work by Cionca, Muller and Mombelli in 2014 illustrates the efficacy of zirconia based implants, which during the study had an aesthetic complication rate of zero percent. The study goes on to discuss how there were no instances of bone loss over two millimetres seen. An

87% one-year survival rate was achieved and that all failures were as a result of aseptic loosening (Cionca, et al., 2014). The article does state however that further monitoring is required to discern the long term survival rate of zirconia based implants in comparison to titanium based implants.

This data may already exist, in work by Zembic et al 2013 in the same journal, which describes a five year randomized clinical trial comparing zirconia and titanium abutments. The study mentions that in both cases, all the patients that were contactable suffered any form of abutment fracture, meaning the survival rate was one hundred percent in both materials. Implant survival was reported to be 88.9% and 90% in zirconia and titanium implants respectively. All mechanical measures, including probing pocket depth, bleeding on probing and plaque control record found no significant differences. Radiological analysis found that bone levels on both the distal and medial sides of the implant were very similar both being within 0.2 units of each other (Zembic, et al., 2013). The works by Zembic et al 2013, and Cionca et al 2014 validate the concept that little to no difference currently exists between titanium and zirconia based implants and as such, one can be used as an alternative to the other. That being said, other considerations such as economic factors should be taken into account. This sentiment is highlighted in a report published in the Australian dental journal by Regina Mericske-Stern 2008, which discusses prosthetic considerations including the socio-economic status of both the patients and the health care system they are within (Mericske-Stern, 2008).

There has also been a small amount of research discussing the effect of laser surface processing on ceramic materials. An article by Kakura et al 2014 investigated the effect of laser treatment of zirconia based implants and evaluated the effect this has on osteointegration and histomorphology. The article reported that the bone implant

contact ratio (BIC) was 1.25 times higher in treated samples as opposed to non-treated samples on cortical bone, the result of which was found to be statistically significant. However, on all other areas the difference in BIC was not significant when compared to normal samples. Removal torque (RTQ) was in excess of seven times higher than the control samples producing a statistically significant result. The article indicates that laser surface modification created a rougher surface profile that was able to enhance osteointegration (Kakura, et al., 2014).

1.2.3.5 Biocomposite Material Alternatives

Bio composite or polymeric hybrid materials are produced via the combination of two or more distinct biomaterials to produce one with intermediate properties drawn its constituents. A good example of which are PEEK biocomposites which are currently being investigated to discern their efficacy and place within the paradigm within material science (Edwards & Werkmeister, 2012)

A paradigm that is regularly described in literature is the use of biomaterials in tissue engineering for bone healing. The modus operandi of tissue engineering approaches are to attempt to generate bone and stimulate healing using a combination of cells and biomaterials. Materials such as collagen and chitin have been demonstrated to have a positive effect on cellular responses and are ideal for use in the body due to their biocompatibility and biodegradability (Swetha, et al., 2010). Chitin based scaffolds show promise and at present has been touted as an alternative to synthetic 3D polymer based materials. Work by Jayakumar et al 2011 such as the work by Li et al which uses PET to make synthetic porous ligaments for ligament replacement. Until recently synthetic scaffolds were the only materials that provided the required properties of allowing cellular infiltration from the surrounding tissue while still providing the correct size and porosity. Recently, chitin scaffolds have been drawing

interest from the scientific community, the ease of processing coupled with the ability to enhance a number of body activities including antibacterial, healing and inflammatory processes, as well as having an increased biocompatibility (Jayakumar, et al., 2011).

Utilizing this alongside Hydroxyapatite (HAP) has been noted as beneficial to cell proliferation and differentiation in a number of different articles. Work by Li et al describes its usefulness in LARS in allowing for cellular infiltration into artificial ligaments and its ability to enhance the rate of proliferation and is integrated on a nanoscale to aid in the mimicking of the microenvironment osteoblasts often find themselves in (Li, et al., 2012)

An example of one of the more recently discussed forms of biocomposites are ones based upon calcium phosphates. A review article by Yoshii et al 2012 discusses the benefits of calcium phosphate (CaP)/polyurethane composites in weight bearing implants within the human body, specifically as alternative graft materials for bone defects. The article compares both HAP and β -tricalcium phosphate (TCP) to calcium phosphate based materials for weight bearing implants. Two different composite implants, comprised of HAP/lysine-triisocyanate-based polyurethane (PUR) and TCP/PUR, were implanted into rats with femoral defects. Results were collected via a combination of biomechanical testing and *in-vivo* testing. The results of the biomechanical testing showed that the presence of PUR improved mechanical properties of the CaP and therefore shows potential for weight bearing implants (Yoshii, et al., 2012) *In-vivo* results were evaluated using X-rays, histological sections and micro-computed tomography (μ CT). The results displayed show that the TCP/PUR mix was able increase cell proliferation and viability relative to HAP as well as supporting osteoblastic differentiation. No adverse inflammatory response was

reported in any of the animals used in the testing process, which was confirmed by X-ray, histological sections and μ CT. Histological analysis also showed evidence of cellular ingress and infiltration (Yoshii, et al., 2012). The article shows that potential superior alternatives to the use of HAP are currently available for use and that not only do they have a beneficial effect on cell responses, they can be easily integrated with both current and potentially future biomaterials just as occurs in HAP.

Not only does the article indicate the efficacy of TCP it also highlights its utility in being combined with other biomaterial to form biocomposite materials. An article published by Patntirapong et al 2014 shows how different combinations of TCP and other biomaterials can have varied effects when undergoing secondary surface processing. The article discusses how the usually lack lustre adhesion characteristics of poly butylene succinate(PBSu) based biocomposites could be overcome using surface modification, in this case roughness modification via hydrolysis using 1.5M sodium hydroxide (NaOH). The article states that of all the different percentage combinations used, 40% TCP with 60% PBSu produced the highest level of roughness. The article then goes on to state how this sample not only provided an improved adhesion and proliferation but also allowed for high levels of vinculin-positive focal adhesion formation (Patntirapong, et al., 2014). This is a key point as vinculin regulates the adhesion process via recruitment and release of core adhesion proteins (Carisey, et al., 2013). The article also demonstrates a number of principles that are key to this study, in that surface modification allows for an increase in cellular roughness and this has a positive effect on cellular proliferation, as opposed to laser surface treatment, hydrolysis was used in this instance.

Another article which touts the benefits of TCP is an article published in 2013 by Tsuzuki et al, which describes the effect of TCP in equine models for osteogenic and chondrogenic healing. Seven thoroughbred horses had either a TCP/gelatine sponge loaded with BMP-2, hMSCs and platelet rich plasma (PRP) or a saline loaded TCP/gelatine sponge inserted into a 10mm diameter hole which had been drilled into the femoral condyles. Results were measured via histological and macroscopic analysis as well as computerised topographical analysis. The results showed that the median subchondral bone density and macroscopic subscores for joint healing were significantly higher in the treatment loaded legs providing results statistically significant (Tsuzuki, et al., 2013). The article also went on to say that the hyaline cartilaginous tissue was observed in a larger area on the treatment based group, however there was no significant histological difference between the two treatment groups. This article not only highlights the efficacy of TCP when it comes to not only bone healing but cartilaginous healing also. One of the main potential criticisms of this article from an outside perspective is the lack of inclusion of a none TCP based baseline, this would have allowed for the evaluation of not only the loaded sponge, but also the effect of TCP which out added benefit of the chemicals loaded into the TCP/gelatine sponge but the article does show the potential benefit of not only in osteogenic healing but in other areas as well.

An article published by Dilber et al 2011 takes the premise of different methods of surface modification further by comparing multiple different surface modification processes on the same type of lithium disilicate based core ceramic disks. In the article, sand blasting using aluminium oxide (SB), SB combined with Er: YAG laser processing (SB-L), Er: YAG (L), 5% hydrofluoric acid (HF) and HF-L. Just as in the study Profilometry was used to evaluate surface roughness as well as using AFM to

further investigate surface profiles. The article showed that SB surfaces had a statistically significant different level of roughness when compared to L and HF surfaces. Tests also showed that the SB and SB-L groups have a statistically significantly higher level of roughness than the other groups (Dibler, et al., 2012). This article indicates that combining processing parameters may produce a roughness value that is superior to the roughness achieved by each method individually. The potential effect of this on cell growth is not mentioned in regards to trends observed in literature, nor is it hypothesised within the article. When trends exhibited in research are considered, an increased roughness should increase cell adhesion and alongside that increase the cell population. This posits a potential future research method based around combining surface processing methods to provide new methods to further improving cell adhesion and proliferation (Hu, et al., 2011).

Both HAP and TCP are mentioned in literature have been highlighted as showing potential for enhancing a number of body processes such as inflammation and antibacterial action, but most importantly cell healing. An article by Ghanaati et al 2012 analysed the *in-vivo* reaction of HAP, TCP and a mixture of the two via subcutaneous implantation in rats for thirty days. The tissue reactions were measured using histomorphometrical and histological methods. The results showed that TCP attracted much larger multinuclear giant cell formations within the implant bed than its HAP counterpart, as well as the fact that the level of implant bed vascularisation was much higher in the TCP beds than the HAP bed. The mixed implant fell in between these two groups. The article does go one to state however that within the first 15 days, high giant cell formation and vascularization rates were observed, which were comparable to the TCP-group. However, after 15 days, the tissue reaction, i.e. the extent of multinucleated giant cell formation and vascularization, was comparable to the HA-

group. In conclusion, the combination of both compounds HA and TCP may be a useful combination for generating a scaffold for rapid vascularization and integration during the early time points after implantation and for setting up a relatively slow degradation. Both of these factors are necessary for successful bone tissue regeneration (Ghanaati, et al., 2012).

Other potential substances exist that are outside of the realm of materials such as TCP or HAP. A study carried out by Martinez et al in 2014 discusses the potential use of deproteinated bovine bone (DBB) in comparison to TCP. The article showed that both materials worked synergistically with the collagen membrane that was placed over the top of the formed defects within the rabbit models employed. The results indicated that while DBB showed rapid bone growth is observed linking the disparate particles, after week 16 the growth within the matrix slowed, partially due to the lack of degradability exhibited by DBB particles. TCP treated areas displayed better bone growth above the dura matter and below the collagen top membrane. TCP also possessed the ability to be reabsorbed allowing for quicker bone growth and healing (Martinez, et al., 2014). This article does open up an interesting question into whether other potential adjuncts are available outside of those previously mentioned, both HAP and TCP have been shown in research to be beneficial to cell growth and their ability to be reabsorbed by the body lends itself to matrices that can be removed or degraded after implantation allowing for much fast bone healing and while interrupting natural bone structure to a minimal degree. Biphosphates such as Zoledronate also offer a potentially beneficial effect when applied to osteogenic growth models. An article published in 2013 by Kadow-Romacker et al illustrates this fact. The article discusses the effect of coating TCP based grafts with zoledronic acid (ZOL). Over the course of the study, poly (D, L-lactide)/TCP grafts were coated with three different

concentrations of ZOL. The article then shows that TCP and ZOL in combination increased the production of osteocalcin and osteoprotegrin as well as sRANKL in osteoblast-like cells (OLC) (Kadow-Romacker, et al., 2013). The polymer materials also caused a dramatic increase on collagen type one and osteocalcin production. The article finishes stating that coating TCP with ZOL has a stimulating effect on osteoblast like cells not only that, but the presence of ZOL also inhibited osteoclastic action. This article points toward biphosphates as having potential applications in bone graft materials (Kadow-Romacker, et al., 2013). The effect of ZOL in the mitigation of osteoclastic action has already been demonstrated as was shown in other studies (Bosemark, et al., 2013). This posits the potential of a biocomposite formed of polymeric based materials, biphosphates and biominerals such as TCP or HAP.

Calcium phosphate based materials have been shown to have a number of potential applications in bone remodelling. An article by Dumas et al. was written in 2010 discusses the potential of injectable allograft made of bone/polymer composites with modifiable properties. At the current time a large amount of research has been targeted towards the subjects of injectable cements with osteoinductive properties. Currently, the vast majority of materials that have been formulated are relatively impermeable to cells and as a result their usefulness is diminished. In the course of the article, an injectable material was comprised of particulate bone fragments and a PUR based polymer composite. The material was shown to have a fairly rapid working and fast drying time were recorded, comparable to calcium phosphate cements, which are currently the gold standard. When applied to femoral plugs in athymic rats, the biocomposite was shown to allow for rapid cellular infiltration, the recalcification of the surrounding matrices and rapid bone healing (Dumas, et al., 2010). This article is interesting in that it highlights potential uses for materials that have established uses

in a novel manner. It also posits questions regarding the use of other materials in a similar manner.

An article published in 2013 by Yu et al further lends credence to the potential of established biomaterials in new treatment modalities. The article discussed the comparison between calcium phosphate cements (CPC) and CPC/Bioglass (CPC/BG) composites. The article discusses a number of points of superiority recorded in CPC/BG as opposed to pure CPC materials. The CPC/BG materials were shown to have greater malleability as well as a slower setting time, the material also included a greater degradability and an improved bioactivity. The article also mentions how the CPC/BG was more able to help produce a HAP layer when submerged in simulated bodily fluids. CPC/BG was also shown to have increased cellular proliferation, attachment and differentiation when compared to CPC (Yu, et al., 2013). This article further illustrates the point that future works can be found and utilised using well established materials in methods for which they were not initially envisioned. The use of CPC/BG based composites could also be further augmented by the application of biominerals such as HAP or TCP. Or the combination of biominerals and biocomposites materials comprised of different material classes.

Polymer-Ceramic (Polyceramic) biocomposites are another class of biomaterial that has potential in augmenting human body processes. Work by Zhang et al 2014 illustrates this point well where it discusses the use of nano scale HAP/polycaprolactone (HAP/PCL) based materials as an osteoinductive scaffold. This scaffold was also coated in dose dependant concentrations of HAP which, as already mentioned has been shown to augment osteoinductive action *in-vitro* and *in-vivo* (Yoshii, et al., 2012). Spiral based HAP/PCL scaffolds were prepared before the experiment using different ratios of HAP and PCL with modified porosities using

techniques that leached salt from the materials. Human foetal osteoblast (hFOB) were cultured for up to 14 days on differing ratios of scaffolds and the results were measured for biocompatibility. Biocompatibility was comprised of a number of different measurements including, adhesion, viability, proliferation, differentiation and genealogical expression relating to bone growth. The results of the article showed that spiral based scaffolds supported proliferation, viability and adhesion in hFOBs, with similar trends to other materials including neat polymeric scaffolds in terms of proliferation (Zhang, et al., 2014). This being said the spiral based scaffolds showed much higher levels of alkaline phosphatase and mineralisation in the surrounding matrix. The most positive ratio of growth in osteoinductive terms was shown to be a HAP/PCL ratio of 1:4 which had higher levels of ALP as well as increased levels of osteogenic related proteins which had been measured semi quantitatively using reverse transcriptase PCR methods.

A number of things can be taken away from this article for one thing the use of semi quantitative methodology does cast a small shadow on the paper in terms of specific trends, however it still allows for the abstraction of generalised trends regarding the efficacy of HAP/PCL biocomposites. Those trends tended to indicate the beneficial nature of Polyceramic composited. HAP's efficacy has been proven on a number of different occasions and the effect of TCP was not explored or posited within the article and this could be a potential area of future research. Different combinations of HAP and or TCP as well as other polymeric materials could also be used as future works within this project or within this area of research as a whole and the outlook for osteoinductive scaffolds and biocomposite cements is an intriguing and positive concept.

While injectable biomaterials have been previously discussed, other methods do offer potential of creating scaffolds and plugs that can be used within the human body, one such method has been dubbed electro spinning. As with the previous article, a biomaterial was formed from HAP and PCL, however in this situation, collagen one was also added. Collagen one has been shown in literature to work synergistically with biomaterials towards bone healing (Kadow-Romacker, et al., 2013). In this article by Phipps et al 2011 a number of different scaffolds were electro spun to include each material independently and separate to one of the other as well as tricomponent scaffolds which contained all three. The results showed that hMSC's adhered better to the tricomponent scaffolds than any of the uni or bicomponent based scaffolds and in the case of the collagen only scaffold, no cell growth or survivability was reported, this may have been related to the elasticity modulus of the material (Phipps, et al., 2011). The article also supposes that the improved response in the tricomponent materials may have been related to increase adhesion protein absorption which was exhibited when the materials were exposed to serum *in-vitro* and *in-vivo*. Tricomponent seeded cells were also shown to exhibited higher expression rates of proteins key to cell survival and osteoblastic differentiation (Phipps, et al., 2011). This article offers a perspective that until recently has been one of the rare in biocomposite research, more than bicomponent composites. Beyond this fact which again is positive as each material was shown to have a beneficial effect compared to each individually, there is not a lot otherwise to make this article unique compared to others that have been previously published however the concept of multicomponent materials is both intriguing and offers hope that other polymeric and multi biomaterial classes are possible.

PEEK based biocomposites have drawn a large amount of attention from the scientific community for their utility in a myriad of different treatment modalities. With the potential to be synthesised in environment specific shapes either via injection of materials or via electro spinning. This could be used to provide improved healing in areas of the body that possess low vascularity or areas where healing may be hindered by other factors. Chief concerns in regards to not only PEEK biocomposites but also injectable materials in general. An article by Page et al 2012 discusses this point and others related to injectable biomaterials as well as PEEK based biocomposites. The article discusses the usefulness of the kinetic model in the prediction of biocompatibility also as it successfully predicted not only porosity but also the manner in which this would affect biocompatibility *in-vivo* (Page, et al., 2012). This article hypothesises a different method of predicting biocompatibility and can be applied to future works.

An article published by Wang et al 2014 which discusses the use of biocomposites comprised of PEEK and nano-fluorohydroxyapatite (nFHA) in comparison to pure PEEK based materials. The PEEK/nFHA based materials were shown to have not only display increased levels of osteointegration but the material also exhibited an increased level of antibacterial activity as a result of the blending method used (Wang, et al., 2014). Not only were different blends prepared for use, different surface roughnesses were also prepared, which allows comparisons for the materials to be evaluated on a number of different levels. The results shown in the article indicate that the PEEK/FHA initial cell adhesion in vitro and proliferation were higher in the PEEK/FHA based materials as opposed to the purely PEEK based material sample. Not only that, but the rougher surface profiles were also shown to increase levels of alkaline phosphatase and cell mineralization, indicative of increased osteointegration

(Wang, et al., 2014). It was also noted that the even the PEEK/FHA as received material was able to prevent biofilm formation and bacterial proliferation. During *in-vivo* test the newly formed bone volume was seen to be higher in the PEEK/FHA materials when measured by 2D histomorphology and 3D microcomputer tomography.

This article gives us a number of points which are not only unique but bode well for biomaterials research, it illustrates that PEEK based biocomposite materials have potential in current treatment paradigms. While the article only makes direct mention of dentistry, the results can very easily be applied to other medical issues that require bone healing or osteointegration. While the article evaluates different roughness profiles, it does not mention cell population and the effect of roughness there in, if it follows the general trends displayed in literature then the cell population could be increased by the roughness (Gittens, et al., 2011). It would be an interesting study to see the effect of laser surface treatment upon PEEK/FHA based material surface profiles in terms of inscribing patterns and if the addition of FHA had any effect on the thermal conductivity of the resulting composite. This article highlights a number of areas of future work not only on the biological side of the subject, but also on the engineering side.

An article Rosentritt et al also discusses PEEK composites, however this is in regards to shear bond strength and the effect roughness has therein. The article describes how PEEK a number of different methods of roughening were applied including particle abrasion and activation of silica-modified aluminium oxide. The article states that the roughness has a positive effect on bond strength with significant differences being found between the different roughening techniques (Rosentritt, et al., 2014). This article offers some alternative methods of roughening surfaces as opposed to the laser surface treatment. When considered that a great deal of literature states that

surface topography has a key effect on cell fate then it is worth investigation to see what differences would be present within these roughening processes as opposed to laser surface treatment. Literature also states that a two stage effect could potentially be created using air – particulate etching with a bioceramic like HAP or TCP. An article published by Dunne et al 2013 discusses the creation of a bioactive coating on titanium surfaces by applying the combination of an abrasive and HAP (Dunne, et al., 2013). The article demonstrates the efficacy of this coating on titanium and this leaves further lines of research open as to the effect of substituting titanium for a polymeric based material or swapping HAP out for another material such as TCP. If trends are followed this could have a greater biocompatibility and be relatively simple to recreate and reproduce.

While Nylon has also been used in a number of biocomposites in literature, an article by Abdal-hay et al 2013 discusses the creation of a biocomposite via the electrospinning of Nylon 6 (N6) and HAP for bone healing. A number of different methods of analysis were used within the study including SEM and water contact angle analysis. The results showed that a range of fibre densities and HAP concentrations could be achieved and that a range of hydrophobicity and hydrophilicity could be achieved via different blend mixes and could be used to make nylon biocomposites suitable for hard tissue engineering (Abdal-hay, et al., 2013). The article also mentions the potential for biomineralisation when the materials were immersed in simulated body fluid. As a result of this the article hypothesises the potential for N6/HAP based biocomposite material. The article has a number of features that make it worth mention, the line of investigation of using in hard tissue engineering which is generally something that Nylon based materials are not suitable for due to their flexibility (Mehrabanian, 2011).

Another point to mention which has been highlighted elsewhere. is the fact that the combination of materials into a composite can result in increased rigidity and via that an improved utility in implants, something that pure Nylon has long been shown to have an issue with. An article discusses this concept and highlights it by measuring physical factors such as fracture resistance and delamination onset in pure materials and electrospun biocomposites. The article discusses how the composites used generally had more robust characteristics than their separate composites, this being said it is possible that a material could become too brittle and have a negative effect *in-vivo* (Shivakumar, et al., 2009). That is not discussed in this article however which is going at the idea purely from a materialistic perspective.

1.2.4 Surface properties

Stem cells are known to take cues from the microenvironment they are subcultured in, and the modulation of this environment can modify cellular responses. Modifying surface properties of biomaterials yields similar results as this forms a significant part of the microenvironment when cells are cultured upon them. The two key properties that can be modified are surface topography and surface chemistry.

1.2.4.1 Surface Topography

Biomaterial topography independent of surface chemistry has been shown to have a substantial effect of cellular behaviour (Martinez, et al., 2009). Oh et al 2008 reported a potential use of topography by showing that stem cell fate could be dictated purely by the alteration of the diameter of nanoscale structures. The article states that changing the diameter and other dimensions of titanium oxide nanotubes resulted for the increased hMSC adhesion or a specific differentiation of hMSCs into osteoblasts as a result of geometric cues despite the lack of osteogenic media present (Oh, et al.,

2008). Carbone et al 2006 discusses the characterization of nanostructured titanium oxide films produced by the deposition of titanium oxide clusters. It describes how the film possesses a granularity and a porosity mimicking those of typical extra cellular matrix (ECM) structures and adsorption properties that will allow functionalization with molecules such as DNA, properties and peptides. (Carbone, et al., 2006).

The article used a number of different cancers and primary cell types were grown upon the aforementioned micro-surface to ascertain their efficacy as a substrate for cell and tissue based applications via bright field microscopy and immunofluorescence. The results suggest that the micro-surface will support the growth of primary and cancer cells without the need for the presence of ECM proteins and is a new material as an optimal substrate with a number of different cell based applications. (Carbone, et al., 2006).

The article reports how topography of a biomaterial can be modified, or created using specific processing parameters to elicit a desired; in this case beneficial, effect upon the stem cells grown in contact with the material. In this case, titanium oxide layers are formed in such a way as to mimic the ECM that the cell would naturally be found in. This is one method through which cell growth can be affected by the modification of the topography of the biomaterials on which it is grown, this is similar to the method used by Chan et al 2013 where the dendritic pattern of the weldment zone allowed for the greater attachment of the cells over the surrounding area (Chan, et al., 2013). This can be seen in figure 5 in how the cells orientate themselves along the pattern in comparison to other samples shown.

In the study, the base metal (BM) refers to sample material that was not affected by the laser in anyway, meaning the natural material structure and as an extension of this

topography, remained unmodified. The heat treated zone (HAZ) refers to an area of the material that was affected by the heat given off as the laser was applied, this generally refers to liquefaction, however no other effects beyond heat were applied. The weldment zone (WZ) refers to the area that was directly under the laser beam itself, this area was subjected to much greater heat as well as potential ablation in some cases.

As can be seen in figure five and six the application of laser processing to the titanium substrate resulted in topographical changes as corroborated by the roughness values shown in figures 3 and 4. The weldment zone (WZ) displays a greater height range when compared to the base metal (BM) and the heat treated zone (HAZ) which show a very similar topographical composition. The article showed that the patterns present in the WZ were beneficial to the cells as it allowed them to anchor more securely to the materials as well as to have a greater level of substrate surface contact (Chan, et al., 2013).

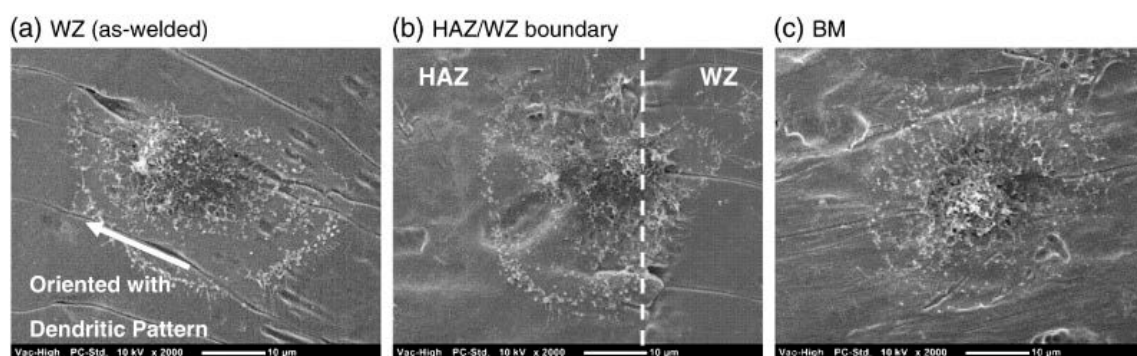


Figure 4: The growth of hMSCs on different Titanium weldment zones. Samples a, b, c show the three distinct surface profiles produced and how the cells adhere to the samples there in (Chan, et al., 2013).

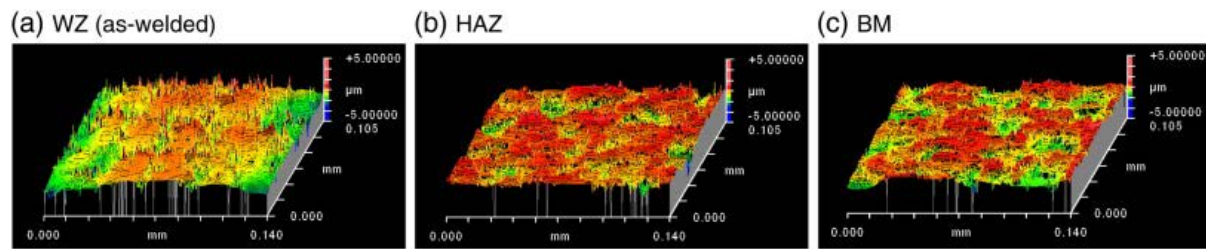


Figure 5: Roughness profiles produced by a White light interferometer. Samples a, b and c show varying levels of roughness ranging from WZ which was roughest to BM which was the smoothest (Chan, et al., 2013).

Figures 7 and 8 illustrate the effect of topographical modification on the adhesion of hMSC cells to the NiTi substrate material. The figures show that the application of surface modification, in this case polishing to produce a smoother surface, resulted in a marked difference in how the cell adhered to the surface features. The polishing showed that in this case, hMSC's preferred a rougher surface profile as this provided more opportunities to anchor themselves more securely as well as have a larger surface to substrate contact ratio. This is further corroborated by the roughness profiles shown in figure 7 and the close up of cell adhesive conformation in figure 8.

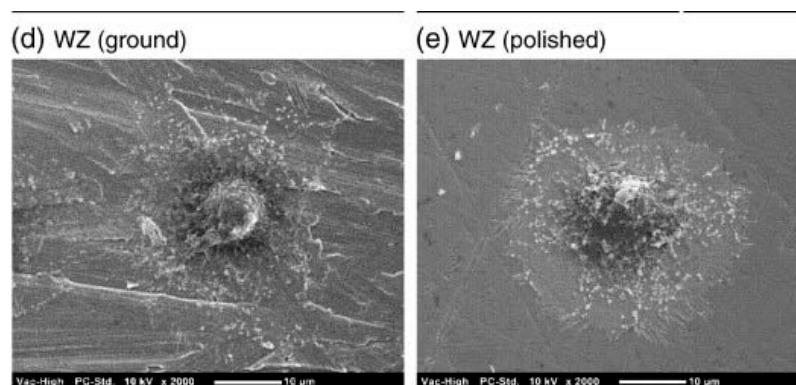


Figure 6: Human mesenchymal stem cells adhering to rough and smooth surfaces. Note how the cells adhere differently to different roughnesses (Chan, et al., 2013)

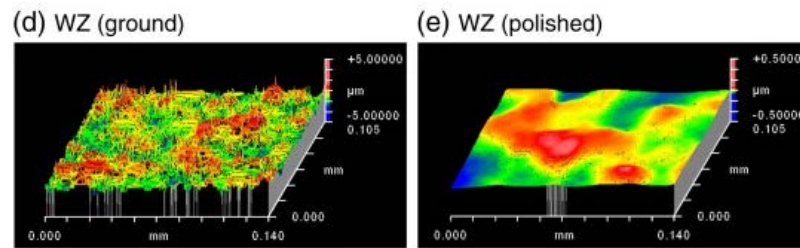


Figure 7: White light interferometry (WLI) roughness profiles of the polished and non-polished surfaces, note the large reduction of surface features in the polished sample (Chan, et al., 2013).

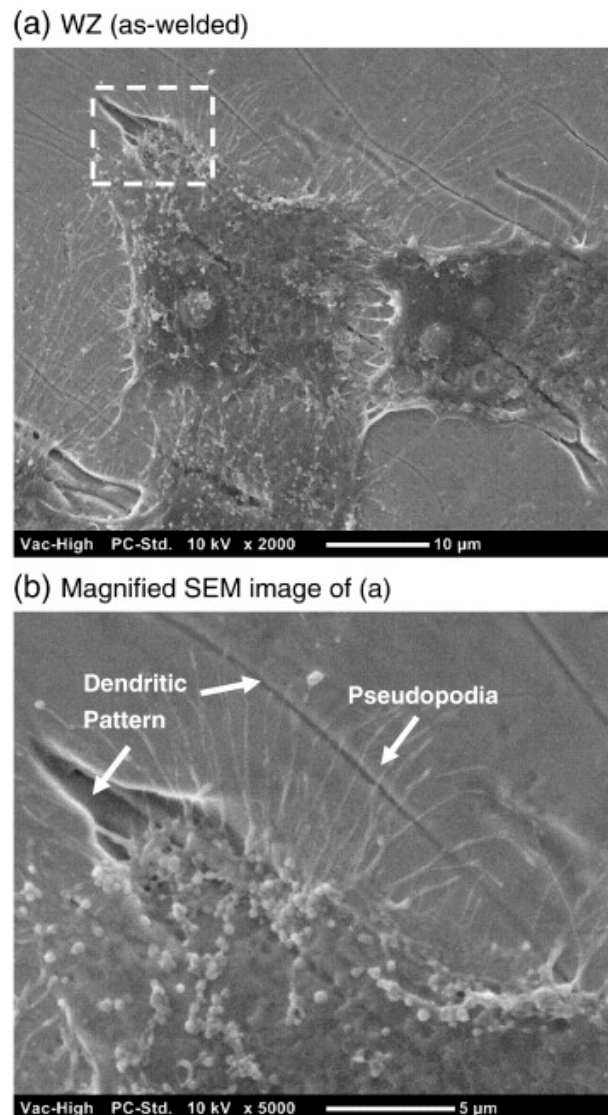


Figure 8: Close up of an hMSC making use of surface features to aid its surface adhesion (Chan, et al., 2013).

One method of modifying surface topography is by simply modifying the roughness of a surface, whether this involves reduction or increase in surface roughness. Wennerberg and Albrektsson discuss the effect that roughness alone has on cell adhesion and osteointegration. As can be expected, there was a demonstrated

correlation between an increase in surface roughness and the level of cell adhesion. However, the article goes on to describe how there is a certain amount of ambiguity as to what is considered the parameters for what is roughness, before deciding that this parameter of surface property is often inadequately or under-reported (Wennerber & Albrektsson, 2009). This indicates that through the course of this study this could be rectified and used as a model for a potential method of standardisation. One potential method this could be brought to fruition is via the identification of a thus far illusive cell surface marker to specifically identify mesenchymal stem cells as mentioned by Kuci et al 2012 (Kuci, et al., 2012).

Work by Guo et al 2007 provides a direct comparison between sandblasted titanium versus smooth surfaces and the effect this has upon osteogenic differentiation and adhesion in MC3T3-E1 stem cells. The results of this study showed that sandblasted titanium oxide surfaces had an increased cellular adhesion as well as increased levels of RUNX-2 and Osterix which are biomarkers indicative of osteogenic differentiation (Guo, et al., 2007). Guo demonstrates how the sand blasted surface of titanium oxide was superior to the smooth machined surface of the as received samples. This is corroborated by Faeda et al 2009 which also discussed the use of laser treatment on titanium within rabbit tibias on both a chemical and a topographical level.

Work by Keselowsky et al 2007 describes the effect of integrin alpha (5) beta (1) ($\alpha 5 \beta 1$) in the regulation of osteoblastic differentiation on roughness surface topographies at the micron-scale. Its efficacy has already been reported previously in literature in regards to the effect of different chemical compositions but in the vast majority of these studies topographical factors were not taken into account (Keselowsky, et al., 2007).

The article discusses the effect of growing stem cells on titanium substrates with three different roughness characteristics, plasma sprayed titanium with irregular projections (TPS), chemically polished titanium (PT) and grit blasted and acid etched titanium (SLA). Cell spread well and had larger nuclei on PT based substrates, yet has a smaller footprint on SLA and TPS substrates. This is most likely due to flatness of the surface allowing the samples to spread out further. Samples on the TPS and SLA based substrates most likely have a similar if not larger level of surface-substrate contact, but over a smaller area due to the irregularities within the topography. This is similar to what is reported in work by Chan et al 2013 which showed that the footprints of the hMSCs was effected by the titanium topography (Chan, et al., 2013).

The results showed that $\alpha 5\beta 1$ binding was higher on the rougher surface profiles, TPS had the highest level of $\alpha 5\beta 1$ binding followed by SLA and finally PT. Focal adhesion kinase phosphorylation is also increased in the same manner, this can be used as a marker of increased cell adhesion and spreading (Michael, et al., 2009). The results of the article indicate that, on the micron scale, surface topography modulates the binding of $\alpha 5\beta 1$ as well as the activation of FAK (Keselowsky, et al., 2007). The article also mentions how the regulation of alkaline phosphatase and DNA synthesis both require signalling via $\alpha 5$ dependant pathways and while this effect is independent of the surface topography, increase levels of $\alpha 5\beta 1$ may have an effect.

1.2.4.2 Surface Chemistry

The surface chemical properties of a biomaterial are crucial to defining not only its general chemical reactivity but also its biocompatibility for use in the human body (Phillips, et al., 2010). Unfortunately, the properties that make the vast majority of biomaterials candidates potentially useful for implantation is their lack of reactivity, also reduces their effective biocompatibility. This means that the body does not react to

them in an optimal manner, thus increasing healing times and increasing the risk of immunological rejection. Even the most inert implantable materials can still suffer from adverse host reactions as the immune system becomes aware to their presence and targets them as a foreign object. This is termed foreign body response (Anderson, et al., 2008).

Work by Thevenot *et al.*, 2008 describes how implants made from materials with hydrophobic properties undergo protein adsorption with hydrophobic protein within the human body. This adsorption occurs as a result of a number of different molecular and chemical processes. These include processes such as hydrophobic interactions, Van der Waals forces and hydrophilic interactions. This results in conformational changes occurring within the proteins structure. Hydrophobic and hydrophilic regions within the protein redistribute themselves to allow them both to be in their optimal positions i.e. hydrophobic farthest from water and hydrophilic as close as possible. These conformational changes result in the exposure of normally hidden protein regions that provoke an immunological reaction (Thevenot, et al., 2008). This indicates that while most materials used within human implants are non-immunogenic, chemically inert and generally non-toxic, adverse immunological bodily responses can occur. Over a long period of time this can result in the eventual formation of a fibrous granuloma around the implant (Kamath, et al., 2008).

However, a number of responses in literature have been highlighted that allow the modulation of the immune response. This is done in an attempt to increase biocompatibility and reduce the likelihood or severity of encapsulation and rejection. One method described in literature is the application of surface coating of a synthetic molecule such as poly ethylene glycol (PEG) or poly vinyl alcohol (PVA), which have been shown to prevent protein adsorption (Larson & Sokolov, 2012). This prevents the

cellular mediators of the immune system triggering an immune response via immunogenic regions of proteins revealed during adsorption. This being said, other material chemical properties such as hydrophilic/hydrophobicity and molecular weight and size are also very important (Gardner, et al., 2013).

Work by Curran *et al.*, 2005 describes the behaviour of stem cells when cultured in contact with silane-modified surfaces. The article examined the effect of modifying surface chemistry modification on hMSC differentiation potential after a weeklong culture on a range of different surfaces and a clear glass control sample. Cell adhesion was measured using lactose dehydrogenase. Cell morphology and viability were measured using calcine, ethidium homodimer, cytoskeletal (F Actin) among others. The article also discusses how different cell morphologies were displayed depending on the chemical composition of the surface on which they were grown. Cells grown on surfaces with chemistry comprised of carboxyl groups were shown to display different cellular morphology as well as fibronectin and vitronectin distributions when compared to amine based surface chemistries. Cells cultured on carboxyl based chemistries also show increased markers of chondrogenic differentiation in the form of increased levels of collagen II expression. This contrasts with cells grown on amine based chemical surfaces which have an mRNA expression profile indicative of osteogenic differentiation (Curran, et al., 2005). Work by Curran et al 2005 highlights the fact that chemical manipulation of a surface upon which stem cells can be grown can, and does affect their differentiation capacity. If this could be successfully applied to materials for implantation it could potentially result in the creation of non-immunogenic biomaterials that can increase the rate of precursor and stem cell differentiation with the purpose of healing at an increased rate. Work of this nature has been attempted with some success, an example of which is work by Richardson et al 2008 which discusses the

use of temperature sensitive chitosan-glycerophosphate(C/Gp) hydrogels in the regeneration of damaged and degenerated intervertebral discs (IVD). The study showed how the culture of hMSCs on such gels resulted in a phenotype similar to articular chondrocytes and nucleus pulposus cells. Standard polymerase chain reaction (PCR) tests also showed a lack of expression of osteogenic markers indicating their efficacy as a potential clinical treatment of IVD (Richardson, et al., 2008).

Surface chemistry is to be implanted in the human body is very important and can be modified to adjust cellular response in a manner that enhances the desired effect of the implant (Roach, et al., 2007). For creating and maintaining uniform and distinct chemical surfaces a number of different methods have been used in previous studies (Thevenot, et al., 2008). Lanniel et al 2011 described the use of hydrogels with varying surface composition as well as rigidity. Antibody staining carried out looking for specific markers related to different differentiation lineages indicated that varying the rigidity and surface chemistry resulted different rates of specific marker expression (Lanniel, et al., 2011). This also highlights the idea that bulk properties can have an effect on biocompatibility in some form.

Work by Chan et al 2013 demonstrated the effect of laser surface processing of a nickel titanium (NiTi) substrate used for hMSC culture. In the article, human mesenchymal stem cells are cultured on material samples taken from three distinct regions categorised after surface processing. One set of samples were taken from the weldment zone (WZ), the area where direct laser contact. A second set were taken from the heat treated zone (HZ), the area directly adjacent to the WZ, where heating occurred without direct processing and a third set was taken from the base metal (BM) zone, the base metal zone underwent no appreciable processing (Chan, et al., 2013).

Table one shows the percentage composition of the different samples as analysed using X-ray photoelectron spectroscopy (XPS). As can be seen in table 1 the nickel titanium ratio is lowest in the as treated WZ followed by the HAZ and the ground WZ. The highest ratio reported was the polished WZ sample and that increased carbon content on the surface is explained as most likely being a common by-product of environmental contamination as the alloy cools after heating.

In the weldment and heat treated zones, the samples underwent liquefaction as a result of the increase in temperature. This caused an increase in the concentration of oxygen in the form of metal oxides at the surface, as well as a reduction in the levels of nickel as shown in table one (Chan, et al., 2013).

Sample	Ni/Ti	Ti 2p	Ni 2p ^{3/2}	O	C	N
WZ (as-treated)	0.031	9.01	0.28	31.78	56.24	2.69
WZ (ground)	0.065	8.65	0.56	28.86	60.31	1.62
WZ (polished)	0.123	8.28	1.02	28.24	58.75	3.71
HAZ	0.047	8.81	0.41	30.61	59.02	1.15
BM	0.08	8.53	0.68	29.14	60.6	1.05

Table 1: The most common elements in both treated and none treated NiTi alloys discerned by XPS (%)

(Chan, et al., 2013)

Titanium nickel ratios are often used as a predictive factor for the release of nickel particles into around an implant. This is important as Nickel and other metals such as Cobalt have known *vivo* reproductive toxicity through disturbing octamer-binding transcription factor 4 (Oct-4) activity in stem cells (Yao, et al., 2014). Other factors are

generally also considered as it has been reported in literature that Ni ions can be released as a result of areas of TiO forming in TiO₂ layers. This molecular variability allows for Ni release due to the presence of the defective sub-oxides (Tian, et al., 2011).

Sample	Ti ⁰	Ti ₂ O ₃	TiO ₂	Ni ⁰	NiO	Ni(OH) ₂
WZ (as-treated)	0	3.73	2.53	9.43	77.11	0
WZ (ground)	3.6	5.76	4.08	9.12	68.71	2.48
WZ (polished)	12	7.18	7.95	9.55	60.15	7.22
HAZ	1.81	4.55	5.6	8.36	71.66	1.3
BM	5.77	8.12	4.96	9.77	67.01	3.87

Table 2: Concentration of key metallic species and molecular compositions present in both processed and non-processed NiTi alloy samples as discerned by XPS (%) (Chan, et al., 2013).

If this line of thought is followed it indicates that the as treated WZ sample is the safest sample for the growth of cells (Chan, et al., 2013). This work draws attention to some key points in that it highlights the potential effect of chemical composition on stem cell differentiation and growth. It has already been highlighted in literature such as in work by Curran et al 2005 which discusses the effect of growing stem cells on chemically distinct different surfaces. Work by Richardson et al 2008 indicates that implanted surface chemistries have potential application in clinical treatments. The manipulation of surface chemistry can also result in the opposite effect. Work by Wang et al discusses the use of gold nanorods coated with a number of different materials and the effects on cytotoxicity therein. Over the course of the study the both positive and negative effects were formed. With interest being paid to nanorods coated in cetyl

trimethylammonium bromide (CTAB) which, due to its bilayer structure, resulted in membrane defects and cell death (Wang, et al., 2013).

1.2.5 Rationale

Laser surface processing offers a few benefits when compared to other methods of surface processing such as localised modification, improved resolution and the ease of repeatability. When applied to polymeric material substrates for stem cell culture, laser surface processing produces surfaces that can be tailored to the requirements of the culture and can be applied in a number of different ways for a number of different fields. That being said the amount of research published, while increasing, is still relatively low. As a results projects such as this can be used to lead into more specific avenues of research. The hypotheses chosen in the project were produced to reflect this. Stem cell growth was used to show the benefit of laser processed surfaces as a substrate and to provide parameters that could be refined in later work in an attempt to elicit a specific response. Expression of BMP-7 was used as a marker to discern if bone growth could be induced without the need for specific differentiatinal cell media and to highlight the applicability of this kind of work to fields such as dentistry and prosthesis.

2.0 Methodology

2.1 Materials

A full table of materials and suppliers can be found in the appendices as appendix one.

2.2 Methods

2.2.1. *In-Vitro* Cellular Experimentation

2.2.1.1 Aseptic Technique

All in-vitro cellular experimental work was performed under strict laboratory safety standards as dictated by the University of Lincoln safety guidelines and Tissue Culture Lab safety protocols. As well as this, all cell culture procedures were performed under sterile conditions within a Class II Biomatt Microbiological Safety Cabinet with seventy percent ethanol in water used through out to further mediate the risk of contamination of the cell culture. In a further attempt to reduce bacterial load and risk of infection, specific lab coats were used throughout at every stage of the process alongside latex gloves and sleeve covers reducing bacterial load to a minimum.

2.2.1.2 Cell Media Preparation

Cells were cultured in the presence of Dulbecco's modified eagle media (Sigma-Aldrich Ltd). This was comprised of 89% Dulbecco's Modified Eagle Media (with L-glutamine) (Sigma-Aldrich Ltd) alongside 10% foetal bovine serum (Sigma-Aldrich Ltd). 1% of penicillin streptomycin (Sigma-Aldrich Ltd) was added to minimise the risk of bacterial contamination of cells in culture. Cells were incubated in a CO₂ cell incubator (Wolf Laboratories Ltd.) within which was maintained a sterile environment

at 37°C with an atmospheric concentration of CO₂ of 5% and 95%, the environment was also kept as humid as possible.

2.2.1.3 Cell Culture and Freezing

Human mesenchymal stem cells (Stem Cell Bank, Japan) contained within a 1.8ml cryotube (Sigma-Aldrich) were removed from a liquid nitrogen containment vessel (Thermo-Scientific) and thawed in a 37°C water bath (Grant) until thawed. At this point the contents of the thawed tube was deposited into a pre-prepared T25 culture flask containing DMEM media. The flask was then placed into a CO₂ incubator (Panasonic) which maintained a temperature of 37°C under standard pressure and an atmospheric content of 5%. Cell media was changed within 24 hours of thawing initially and then as required, the maximum time between media changes was 48 hours. Flasks were checked at least once in a 24-hour period to ensure that the cells were under optimal growing conditions. Cells were subcultured at approximately 80% to 100% confluency or after approximately 5 days' culture within a t25 culture flask. Cells were retrieved using a mixture of 0.25% trypsin and 0.02% EDTA in water. Upon retrieval, cells were transferred into a 15ml Falcon tube and topped up with DMEM media. Tubes were then centrifuged at 1200 rpm for 12 minutes at room temperature (~22°C). Once centrifugation was complete, the supernatant was discarded and 1ml of fresh DMEM media was added to the tube. The cell pellet at the bottom of the tube was then resuspended by pressing and depressing the plunger to agitate the media breaking up the pellet and resuspending the cells. 10µl of the cell suspension was placed into an Eppendorf tube with an identical amount of filtered trypan blue to make a 1:2 cell dilution mixture. This was then further agitated to aid mixture before 1 µl of the solution was drawn and inserted into a haemocytometer using a micropipette. The loaded haemocytometer was then placed onto an inverted white light microscope and the cells

were counted using the 4 x 4 grid inscribed on the glass of the haemocytometer. The cells are counted and divided by 4 before being multiplied by 2 and then multiplied by 1000 to get the cell concentration per ml. See equation 1.

$$\text{Number of cells } (n) = \left[\left(\frac{\text{Haemocytometer cell count } (N)}{4} \right) \times 2 \right] \times 1000$$

Equation 1: How to estimate the number of cells per ml of cell media in cell solution

This value then dictated what was done next with the cell suspension. Provided adequate stocks are present, the remaining cell suspension was then seeded into the appropriate number of T25 flasks for cell culture and expansion generally at density of $\sim 7.5 \times 10^4$. For experimentation, cells were 96 microwell plates as in the presence of processed and unprocessed biomaterial samples at a density of $\sim 7.5 \times 10^2$. If cell reserve stock was to be created, 1ml of cell suspension was pipetted from the Eppendorf tube into a Nunc-cryotube (Thermo Scientific). 100 μ l of dimethyl sulfoxide (DMSO) was then added to the cryotube which was then topped up with 700 μ l of FBS. The sample was then mixed before being placed into a Mr Frosty™ Freezing Container (Thermo Scientific) in an Ultra-Low Temperature Freezer (New Brunswick Scientific) set to 193.15K. Cryotubes can be left in the freezing container for up to 6 months, however generally tubes were transferred to a liquid nitrogen cell storage tank after a week for long term storage.

2.2.2. Laser Surface Processing

Two polymeric biomaterials were selected to be the subject of research within this project, the first that was selected was Poly ether ether ketone (PEEK) which had a thickness of 1.5 mm (Goodfellow Corp, USA). The second material selected for use was Nylon 6'6 which had a thickness of 0.5 mm (Goodfellow Corp, USA).

2.2.2.1 Pre-processing preparation

Prior to processing, the as received material samples were cut into 2cm² coupons. The coupons were handled using latex gloves throughout to prevent surface contamination. The coupons were immersed in distilled water for two minutes before being immersed in pure propyl alcohol for 3 minutes while immersed, the samples were ultrasonically cleaned followed by an immediate immersion in distilled water for around 5 minutes to help remove any grease and or residues from prior material processing. Samples were then dried thoroughly in air and using blue tissue prior to laser treatment.

2.2.2.2 Material processing

A 25-watt carbon dioxide based infrared laser marker (Synrad Inc., USA), with an operational wavelength of 10.6 μm was used to treat the material surfaces under standard atmospheric conditions. Processing parameters were modified depending upon the desired pattern, and the distance between the lines. Two patterns were selected for use due to their simplicity and efficacy. Pattern one was a cross-hatch based pattern made up of two sets of lines set that intersect each other at ninety degree angles. Pattern two was comprised of one set of parallel lines set at regular distances from each other. In both patterns the spacing between the lines was varied at regular intervals depending upon the sample, these intervals were 50 μm , 100 μm and 150 μm . A percentage of the lasers 25 W was applied which also was varied as per experimental protocol, powers of 50% (12.5w), 25% (6.25w) and 12.5% (3.125w) were used on both materials in the creation of pattern one. Powers of 25% (6.25W) and 12.5% (3.125w) were used in the creation of pattern two, this was in part due to the potentially negative effects relating over exposure of the material to infrared light. Two traverse speeds were used in the creation of both patterns, 250mm/s and 500mm/s.

2.2.2.3. Post Processing

Upon completion of laser treatment, samples were cut using a mechanical press to produce five-millimetre diameter discs from the laser treated material area. This was done so as to fit the material into the wells on the 96 well plates that were to be used later in the study. Before samples were placed upon a the 96 microwell plate, they were visualised using an optical microscope (Olympus, BX51; Nikon Eclipse TS100) to ensure that the areas cut only contained laser treated surface and to ensure there were no obvious defects that may affect the growth of the cells. After it was ensured that only laser treated material surface was present, the coupons were placed into sealed containers for *in-vitro* experimentation.

2.2.1 Material Designation

Material designations were produced based on the processing parameters used to produce the material surface. These were varied based on percentage power, traverse speed and spacing to produce varying levels of laser power applied per sample. Material designations will use the following format, M1-N for a nylon sample and M1-P for a PEEK used producing the processing parameters of designation 1. This will continue throughout i.e. M2-N, M2-P etc.

Material Designation	Processing Parameters		
	Percentage Power (%)	Traverse Speed (mm/s)	Spacing (µm)
M1	25	250	50
M2	25	500	150
M3	50	250	50
M4	50	500	150
M5	12.5	250	50
M6	12.5	500	150
M7	25	250	50
M8	25	500	150
M9	12.5	250	50
M10	12.5	500	150
AR	0	0	0

2.2.4 Material Wettability Analysis

Processed material samples were removed from their covered container and placed onto the stage in the centre of the Goniometer. A large light was then shone on the sample and a camera focused across the surface. At this point a small mechanical pipette is lowered so as to just in the top of the frame and a drop of distilled water onto the surface of the processed material. The contact angle was then measured using the camera and another drop was then added and the angle taken again. This continued until 5 angles were taken.

2.2.5. Surface Roughness and Topography Analysis

The surface profiles of the two biomaterials used in the study were visualised using 3D Profilometry at the University of Chester (Talysurf Series 2, Taylor Hobson). The roughness (Sa) and maximum peak-to-valley height parameters for each sample were both determined using Tallymap Gold visualisation software which created a visual surface profile as well as the individual Sa values. The mean and experimental standard deviation of the roughness parameters were obtained from five measurements over the surface of the material.

2.2.6. Surface Chemical Compositional Analysis

The chemical composition of the laser treated material surfaces was analysed using X-ray photoelectron spectroscopy at the University of Chester (XPS) (PHI5600, Physical Electronics, Inc.) with a take-off angle of 45° normal to the sample surface. The X-ray source was monochromatic Al K alpha (15 kV, 25 W) and the beam size was 100 µm in diameter. The pass energy for survey scan and narrow scan spectra were 187.5 and 58.7 eV, respectively. The results were then tabularised and graphs of Carbon bond concentrations formed.

2.2.7. Scanning Electron Microscopy

After 24h, 48h to 96h of cell culture, the morphology of the attached cells was examined via SEM in the secondary imaging (SI) mode and the following procedure was undertaken to produce a sample that was dehydrated ready for gold sputter coating. After removal of the culture media, the samples were rinsed with phosphate-buffered saline (PBS) (Sigma-Aldrich, Ltd.) as to remove unattached or dead cells. Adherent cells were fixed with 1.2% glutaraldehyde in distilled water (Sigma-Aldrich, Ltd.) at room temperature for 1hr within a class II biosafety cabinet. After an hour, the glutaraldehyde solution was removed and the fixed cells were washed with PBS prior to dehydrogenation using a graded series of ethanol/distilled water mixtures of 50%, 80%, 90%, 95%, 98% and 100%. Each sample for approximately 10 minutes in each of the graded mixtures and dried in air. The samples were subjected to gold sputter coating for cell morphology observation by SEM. Cell density/cm² was determined following each time course incubation. This was accomplished by analysing the cell coverage on each sample using both SEM and optical micrographs with the ImagePro software. The optical micrographs were obtained using an up-right optical microscope with magnifications varying between $\times 100$ and $\times 500$.

2.2.8 Cytotoxicity Testing

2.2.8.1 Reagent Preparation

A 12mM stock solution was prepared with an MTT concentration of 5mg/ml. Mixing was ensured by vortexing the sample until fully dissolved. This was then aliquotted into 5 separate Eppendorf tubes containing 1ml of mixture each.

2.2.8.2 Cytotoxicity Assay

Cells were cultured on processed biomaterial samples with an initial Culture media was removed and was then replaced with 100µl of fresh media. To this 10µl of the previously prepared 4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) stock solution was added to each well including both a positive and negative control well. The positive control was created by adding the stock solution to a well containing hMSC's grown in the presence of unprocessed biomaterials. Cells were then wrapped in an opaque material, in this case tin foil, and placed back into the cell culture incubator for 4 hours. After which all but 20µl of the MTT/cell media mix was removed and 50µl of DMSO solution was added to act as a solubilizing agent. The samples were then incubated for 10 minutes after which samples were mixed once more before being measured in triplicate at 540nm.

2.2.9 Protein Expression Assay for Bone Morphogenic Protein 7

2.2.9.1 Assay Reagent Preparation

Concentrated Assay Diluent was diluted by adding 20% concentrated diluent to 80% sterilised deionised water to produce a 1:5 dilution mixture this was done in and stored in 50ml falcon tubes. As required, samples were diluted with 50% of the

Samples were diluted with assay diluent two fold to produce a one to two dilution.

- I. 400µl of diluent was added to the provided vial of lyophilized human BMP-7 protein standard to prepare a 50ng/ml standard. 80µl of this standard was then added to 586.7µl of assay diluent to produce the 6000 pico-gram per millilitre (pg/ml).
- II. Wash buffer was produced throughout the experimental process by diluting the provided 20-fold concentrate to standard concentration.

- III. Antibody detection concentrate was produced by adding 100µl of assay diluent to biotinylated human BMP-7 detection antibody. This was then diluted 80 fold with assay diluent
- IV. HRP-Streptavidin concentrate was produced during the experimental process to ensure its efficacy. The concentrate was diluted 400 fold with assay diluent. E.g. 37.5µl of concentrate of 15ml of antibody diluent.

2.2.9.2 BMP-7 Protein Expression Assay

The reagents were removed from a freezer and allowed to come up to room temperature (~22°). 100µl of the pre prepared standard were added to the test wells to produce a serial dilution series ranging from 6000 pg/ml to 0 pg/ml. Wells were then covered and placed on a rocker and left over night for twelve hours at ~4 °C. After twelve hours the solution was removed from the wells and were then washed with pre-diluted wash solution. This was done by adding 300 µl of wash buffer to each well and then removing it, this was repeated four times to ensure that the wells were adequately washed. Plates were then inverted and blotted against a dry paper towel. 100µl of biotinylated detection antibody (bda) was added to each well and then incubated at room temperature for around 1 hour with gentle shaking. After one hour the bda solution was removed and the wells were then washed with 300µl of wash buffer again four times and then blotted using dry paper towels. At this point 100µl of HRP-Streptavidin solution was added to each well and the samples were then incubated for around 45 minutes at room temperature with gentle rocking. The solution was then discarded and the samples washed as has mentioned previously before being blotted and dried on dry paper towels. At this point 100µl of ELISA colorimetric TMB reagent

was added to each well, the samples were then incubated for around 30 minutes at room temperature in dark conditions with gentle shaking. After 30 minutes, 50µl of Stop Solution was added to each well and the samples were read at 450nm immediately.

2.2.10. Statistical analysis

The significance of the observed was studied using SPSS software version 21 (SPSS, Inc.). Probability thresholds were set to the standard 95% confidence value.

3.0 Results

3.1 Stem Cell Growth

Throughout the entire project the hMSC's used grew well and without incident. There was no contamination and passage number was kept low to prevent genetic drift within the cell lines.

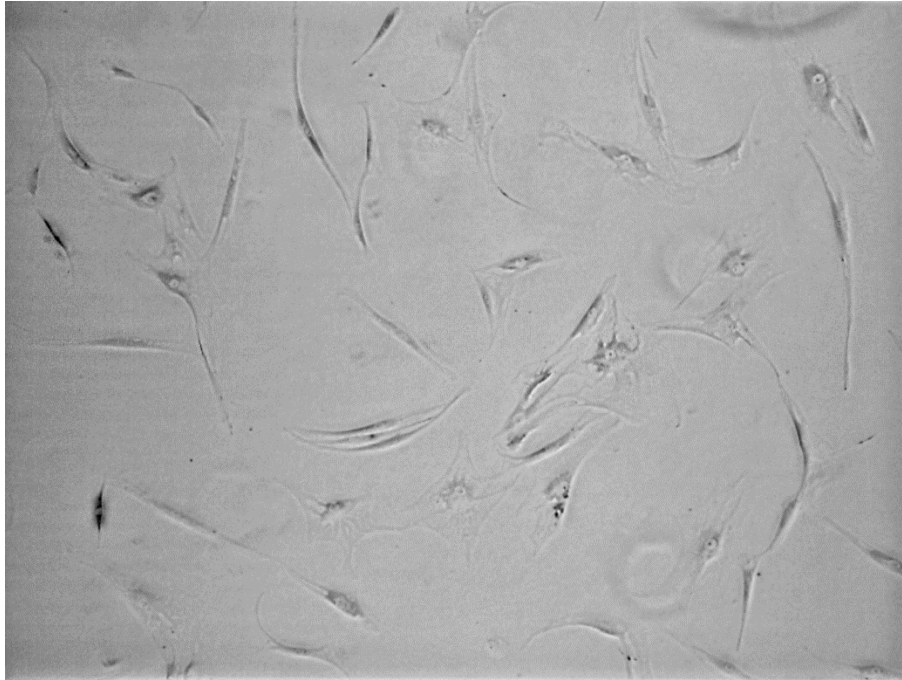


Figure 9: Optical microscope image showing the spindle like structure of hMSC's in culture within 24 hours of subculture (10x magnification)

3.2 Material Wettability

The wettability of the laser treated materials was measured using ultrapure water applied using a goniometer (Ramè-hart instrument Co). Both contact angles and surface energy were measured as a measure of biocompatibility via cell adhesion.

3.2.1 The Wettability of Laser Modified PEEK

Table 3 shows the average observed contact angle of distilled water on each of the different surface samples produced by the various processing parameters. In this

experiment each reading is an average taken from a minimum of five results, an as received sample was used as a control.

PEEK	Contact Angle
AR	70.24
P1	125.03
P2	84.65
P3	132.83
P4	78.89
P5	72.58
P6	61.88
P7	88.14
P8	96.37
P9	69.46
10	68.91

Table 3: A table showing the average of the contact angles recorded on each of the samples post processing with an unmodified as received sample acting as a control.

An as received (AR) PEEK sample was used as a control sample to indicate the expected contact angle on an unmodified sample, as can be seen in figure 10, the average observed contact angle on the AR sample was 70.24°.

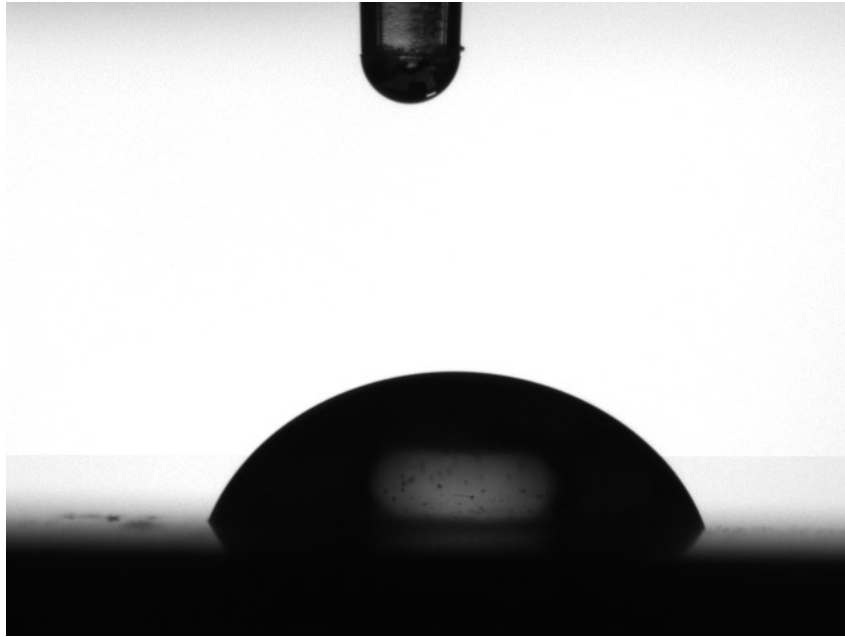


Figure 10: Water droplet on the PEEK control sample with a contact angle of 70.24°

The highest contact angle observed on the PEEK samples was 132.83° on sample P3, this profile was produced under 50% power (12.5W) at 250mm/s with a spacing of $50\mu\text{m}$ spacing between the trenches. It was so hydrophobic that two drops were required to get the droplet to leave the tip of the water dispenser and stay attached to the surface.

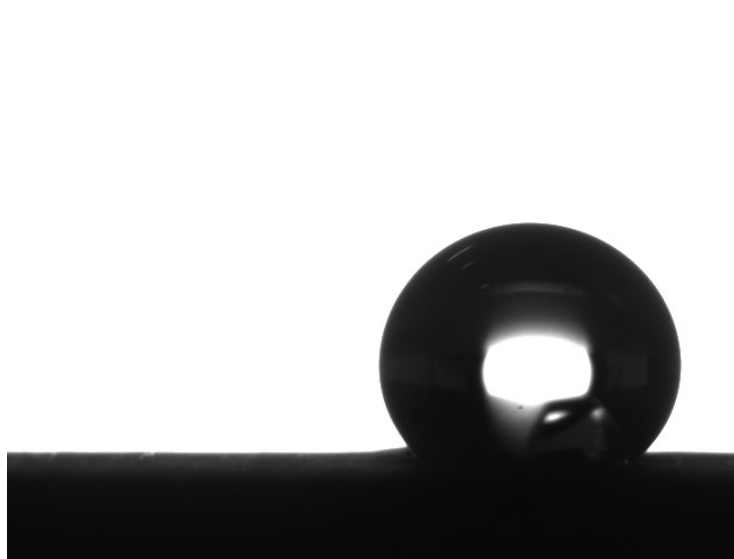


Figure 11: Water droplet with a contact angle of 132.83° on the PEEK P3 sample

The lowest contact angle observed was found on sample P6. This was done at 12.5% power (3.125W) with a traverse speed of 500mm/s and with a spacing of 150 μ m. This sample produced a contact angle of 61.88° which is shown in figure 12.



Figure 12: Water droplet on PEEK P6 which produced a contact angle of 61.88°

Three of the observed contact angles were lower than the angle reported for the unmodified control sample. The remaining seven samples were all higher than the as received sample. The lowest observed contact angle was 8.36° lower than the as received sample. The highest observed contact angle on the other hand, was 62.59° higher than the as received sample. Table 3 indicate that an overwhelmingly hydrophobic effect was elicited in PEEK due to the application of the laser processing used.

3.2.2 The Wettability of Laser Modified Nylon 6'6

Table four shows the average results of the wettability assay performed on both processed and non-processed Nylon 6'6 samples. Sample N3 was unsuitable for testing due to heat contracture and as a result a zero value was prescribed. An as received sample was used a control which underwent no processing.

Nylon 6'6	Contact Angle
AR	53.54
N1	40.43
N2	77.74
N3	No reading
N4	55.00
N5	52.70
N6	60.89
N7	57.97
N8	65.48
N9	43.54
N10	62.01

Table 4: The average wettability of laser modified Nylon 6'6 as measured via surface contact angle using deionised water.

Figure 13 shows the as received Nylon sample which was used a control sample to produce a baseline contact angle on an unmodified material sample the average observed contact angle was 53.54°.

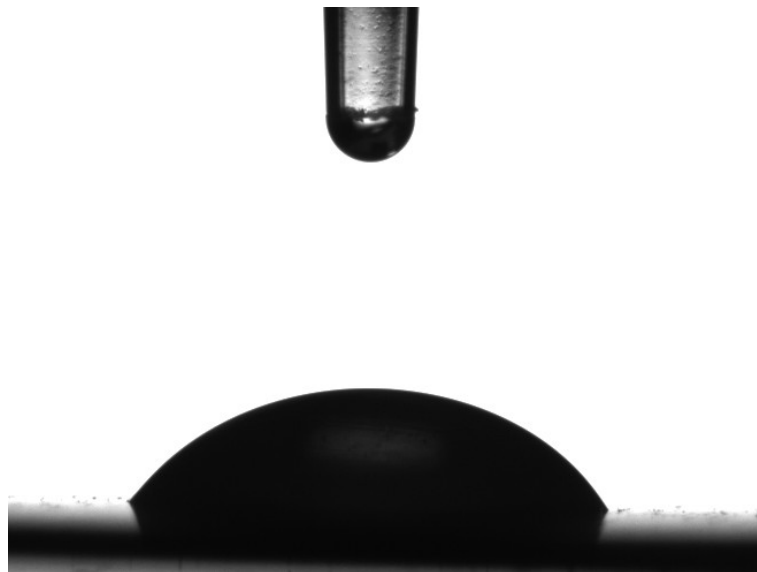


Figure 13: Water droplet on AR which produced a contact angle of 53.54°

The highest observed contact angle was observed when water droplets were applied to sample N2, this sample was processed using a 25% power (6.25W) with the traverse speed set to 500mm/s and a spacing of 150 μ m between the laser contact vectors. The figure shows this sample and the produced contact angle of 77.74°.

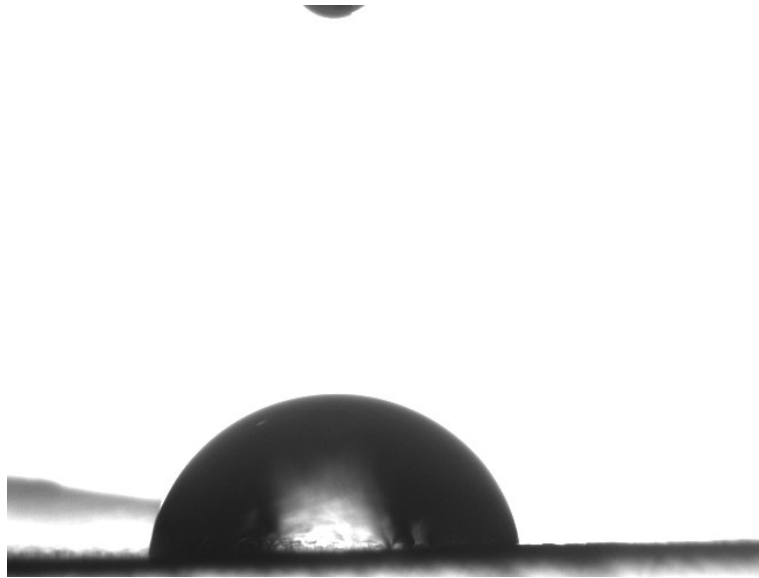


Figure 14: Water droplet on M2 which produced a contact angle of 77.74°

The lowest observed contact angle was on sample N1. This sample was processed at 25% power (6.25W) with a traverse speed of 250mm/s and a spacing of 50 μ m spacing between the trenches. The contact angle produced was 40.43°.

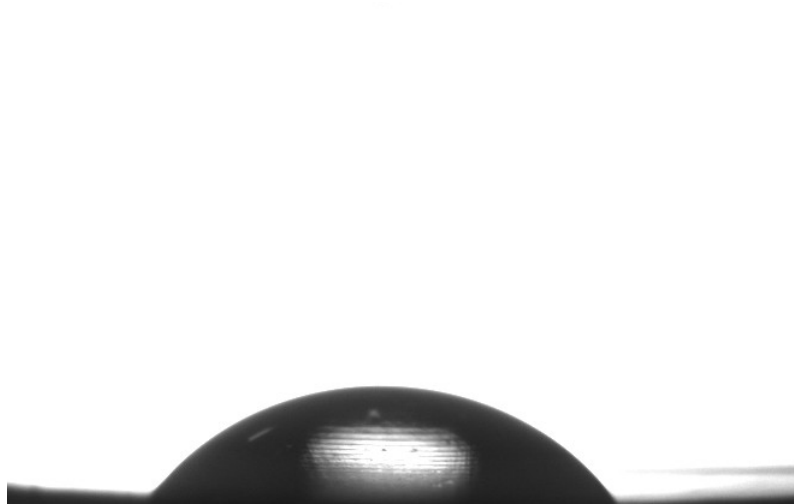


Figure 15: Water droplet on sample N1 which produced a contact angle of 40.43°

Three of the observed samples had a lower contact angle than the as received sample and fifteen were above. However, the highest observed contact angle was still much lower than the highest observed contact angle or surface energy of the PEEK samples. This indicates that the laser treatment of Nylon had a different effect than was observed in PEEK, making Nylon 6'6 more hydrophilic in opposition to PEEK.

3.2.3 Comparison of Wettability between PEEK and Nylon 6'6

A number of conclusions can be made based upon the preliminary data, firstly that the PEEK samples used in the experiment were more hydrophobic than the Nylon and in most cases the values don't even come close to intersecting with each other. Another observation is that when the spacing between the lines and the traverse speed were increased, it resulted in the PEEK surfaces produced becoming more hydrophilic, whereas the opposite trend was observed in the Nylon samples. While discounting the Nylon-M3 sample, which was unreadable due to warping caused by heat contracture, it seemed that a samples with a higher application per square cm² were found to be more hydrophobic.

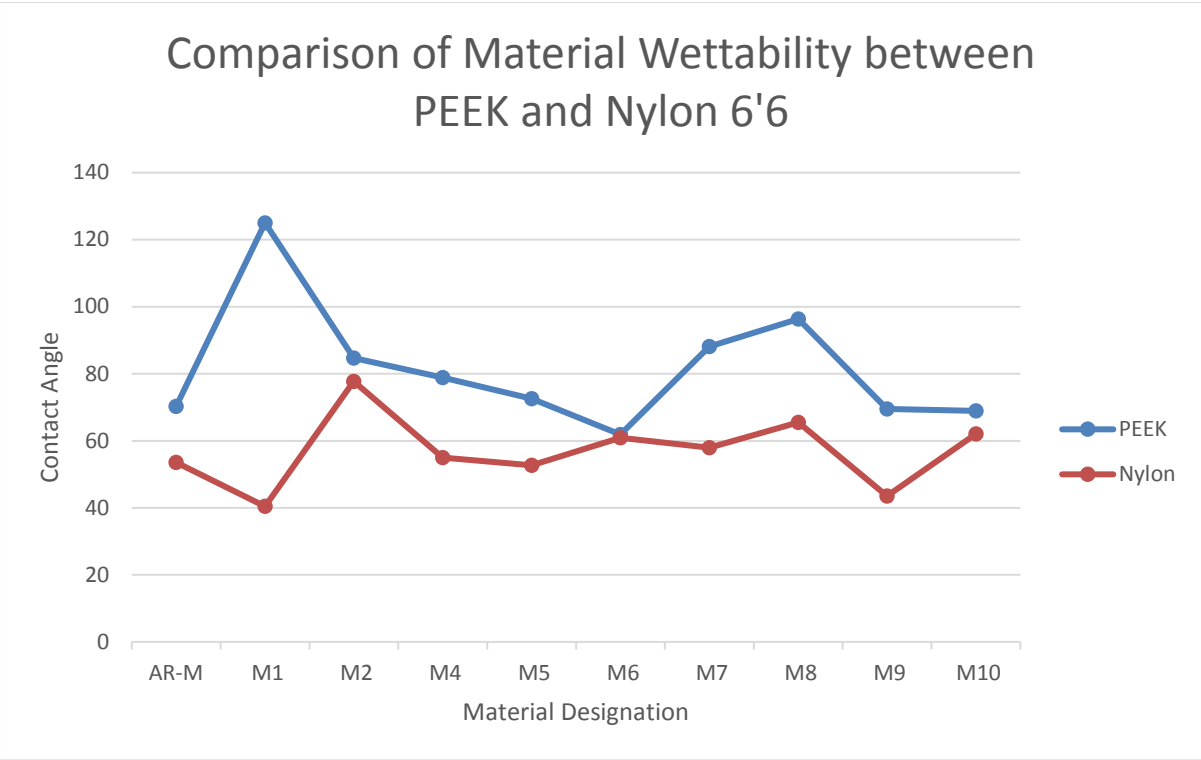


Table 5: A comparison of the different wettability characteristics of all the laser modified materials as observed via wettability. M3 is not present to experimental constraints resulting in lack of data generation.

3.3 Surface Roughness

The surface roughness of the processed samples was carried out by 3D Profilometry (Tallysurf 2, Taylor Hobson) using Tallymap Gold software. An average roughness value (Sa) was taken for each of the treated samples as well as a 3D rendering of

each sample. This is so that the effect of the different processing parameters on surface roughness can be measured. Table 5 presents the roughness measures found, with an as received sample used as a control.

Materials	PEEK	NYLON
AR	4.27	1.77
M1	10.3	14.7
M2	7.98	1.56
M3	24.8	24.8
M4	59.8	4.62
M5	34.6	1.69
M6	5.17	4.73
M7	1.43	3.31
M8	18.4	43.2
M9	9.44	3.85
M10	10.1	22

Table 6: Average roughness values of PEEK and Nylon including an as received control reading

PEEK surfaces with a line based patterns resulted in an increased surface roughness when compared to the Nylon samples. It is also worth noting that the highest roughness values were observed in the lower laser powers in PEEK. In Nylon the opposite is true and the highest roughness values were exhibited in the cross hatch patterns. Table 6 shows the different processing parameters used on the material surfaces resulted in a wide and varying range of surface roughness values.

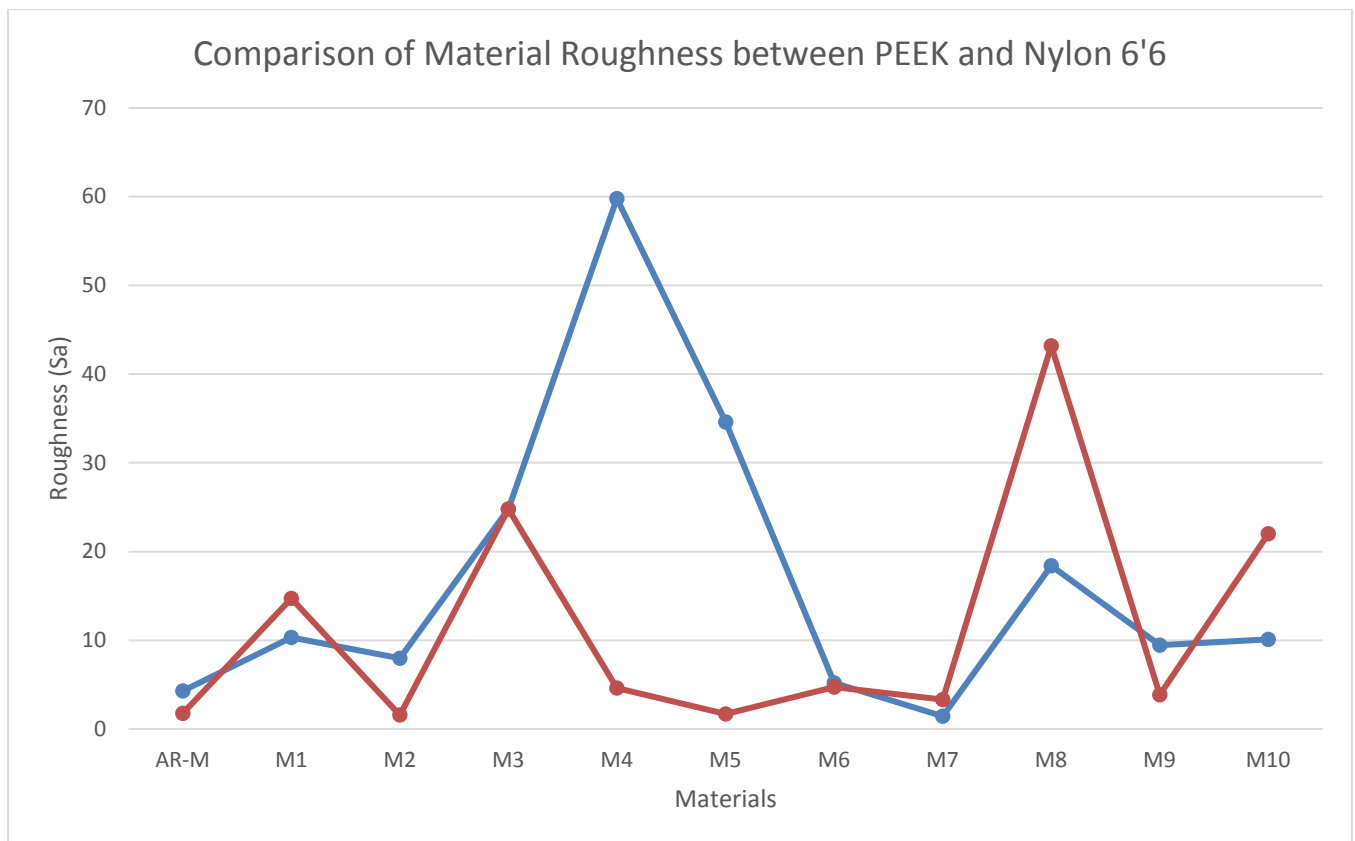


Table 7: A comparison of the roughness values of the two material samples found +/- SE (N=11 per material)

3.4 Chemical Analysis

3.4.1 PEEK surveys and quantification

A similar trend was exhibited on all the material processing parameters used. This could be due to the supb par thermal condicitivity PEEK has. This results in the bonds between the atoms breaking resulting in disintergration as opposed to liquefaction, which would allow for the atmosphere to infiltrate the material surface. In tables 7 and 8, the highest carbon concentration was noted on sample M1 and the lowest was noted on the control.

Name	AR-P	M1-P	M2-P	M3-P	M4-P
C	82.35	90.03	87.15	93.2	86.63
O	13.38	8.06	12.43	5.39	12.74
N	1.28	0.94	0.25	0.73	0.34
Si	0.88	0.39	0.39	0.24	0.17

Table 8: Percentage composition of elements in processed PEEK samples

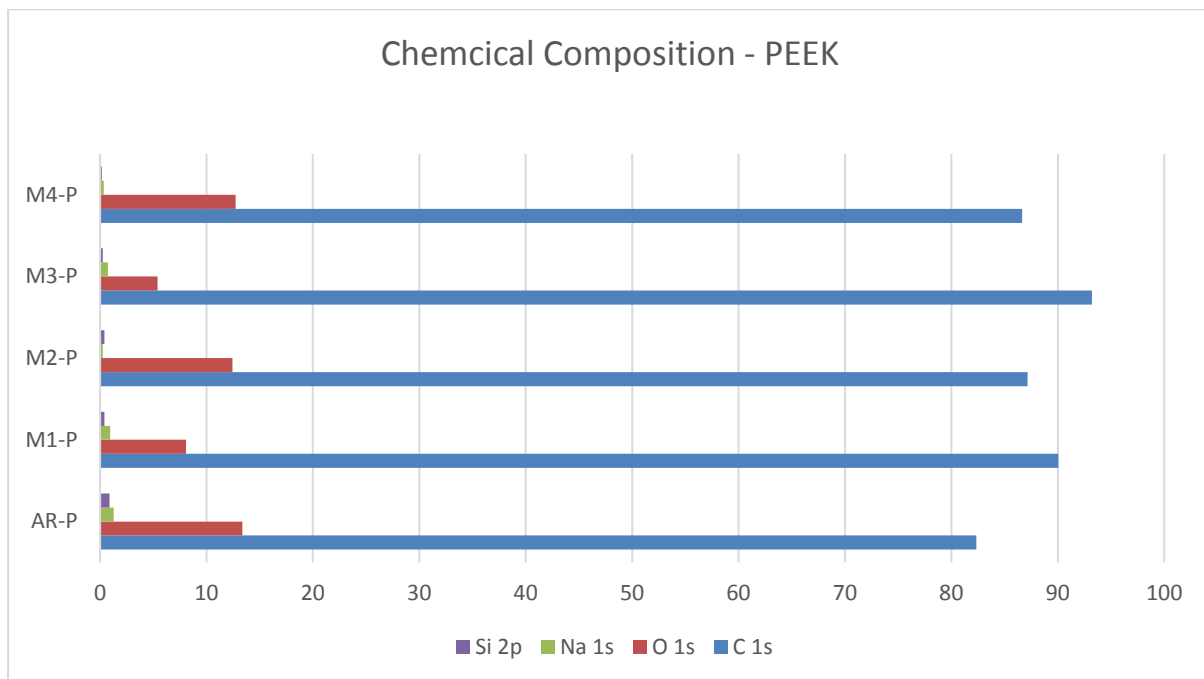


Figure 9: Graph showing the percentage elemental composition of processed PEEK sample

3.4.2 Nylon survey and quantification

The tables again show the percentage elemental concentrations in a Nylon control sample as well as processed samples. The general trends are as expected in all cases the major difference from the control is the increase in the level of nitrogen and a reduction in the percentage of carbon. This could be due to Nylons good thermal conductivity which results in the heat being spread over a larger area, resulting in less bond destruction and liquefaction as opposed to disintegration. This allows for the infiltration of atmospheric elements, chief among which is nitrogen as would be expected, especially as it makes up the majority of the atmosphere.

Element	AR-N	M1-N	M2-N	M3-N	M4-N
C 1s	82.47	77.68	79.41	79.55	83.52
O 1s	11.12	11.14	11.42	11.03	10.9
N 1s	4.56	10.77	8.37	8.86	3.32
Si 2p	0.58	0.3	0.7	0.51	1.48

Table 10: Percentage concentrations of the main elements found in processed and non-processed Nylon

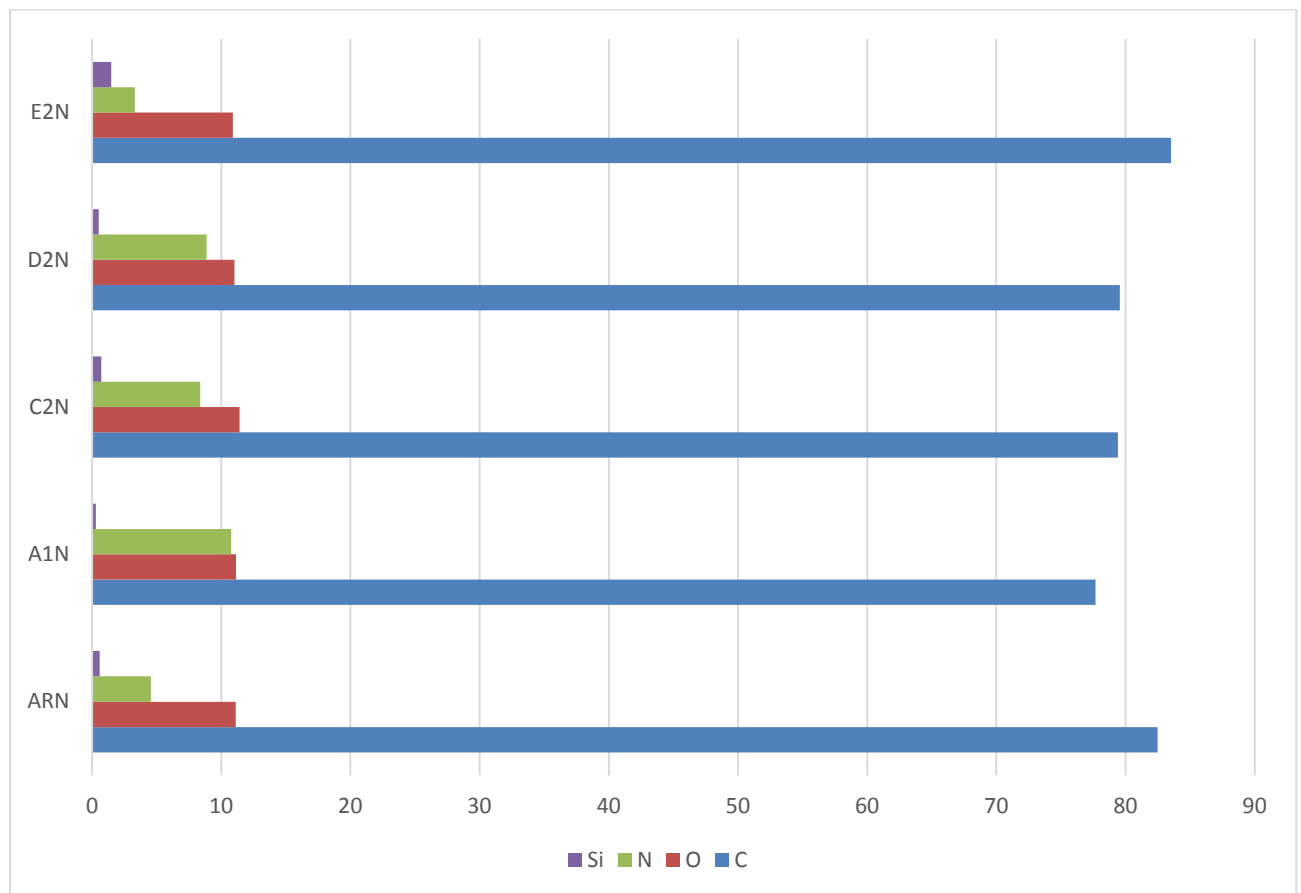


Figure 16: Graph showing the percentage elemental composition of processed Nylon samples and the effect of laser surface processing.

3.4 Cytotoxicity Results

3.4.1 Cytotoxicity Nylon

The result of the Nylon MTT assay demonstrates that as incubation time of the hMSC's increases the cell population increases. While there were no cases where the 24-hour

incubation was higher than the 48 or the 4 day MTT, there were occasions where both the 48 hour and the 4 day MTT were both recorded the highest average result. The 48 hour results were higher in samples A1, C2, F2 and O2 and the 4 day results were higher in samples D1, G1, I2, J1, L2 and M1. In the majority of cases were higher than the higher power lined patterns, and the higher 4 day results were generally observed on the crosshatch patterns, though D1, which was a lower powered line pattern did display a higher average result though the margin between the two was small.

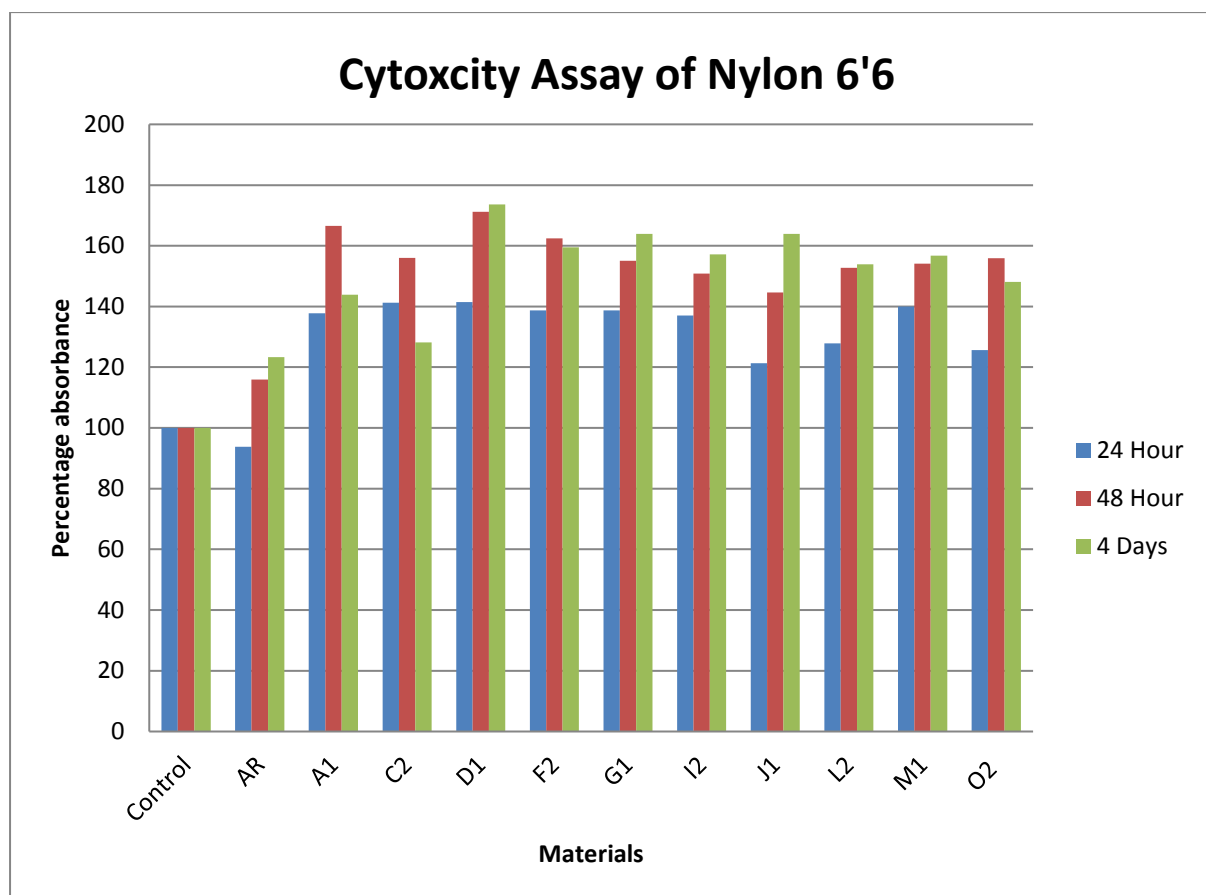


Figure 17: MTT Nylon results with all three incubation times (24 hrs, 48 hrs, 4 days). The effect of longer incubation time was found to not be uniform in all cases and in some cases resulted in a lower level of population growth over time as opposed to an increase.

Pairwise comparison was used to determine if there was any statistical significance between the different incubation times within the material groups. The results showed that there was a significant difference between the 24-hour incubation and both the 48

hour and the 4-day incubation, results showed that this result was statistically significant. While there was a difference between specific cell populations when grown on some of the materials, over all the difference was not found to be statistically significant.

3.4.2 Cytotoxicity PEEK

The results of the MTT assay in the presence of PEEK indicate that as the incubation time was increased, the cell population also increased, in all instances the presence of biomaterials improved this dramatically. In contrast to the nylon MTT, there were a number of instances in which the 24 hour MTT reading was higher than the 48 hour reading, these occurred at C2, I2, L2, M1. There were no instances in which the 48 hour MTT was higher than the 4 day readings however, in every result the 4-day incubation elicited the largest amount of formazan, indicating that cell population occurred in the longer incubation times.

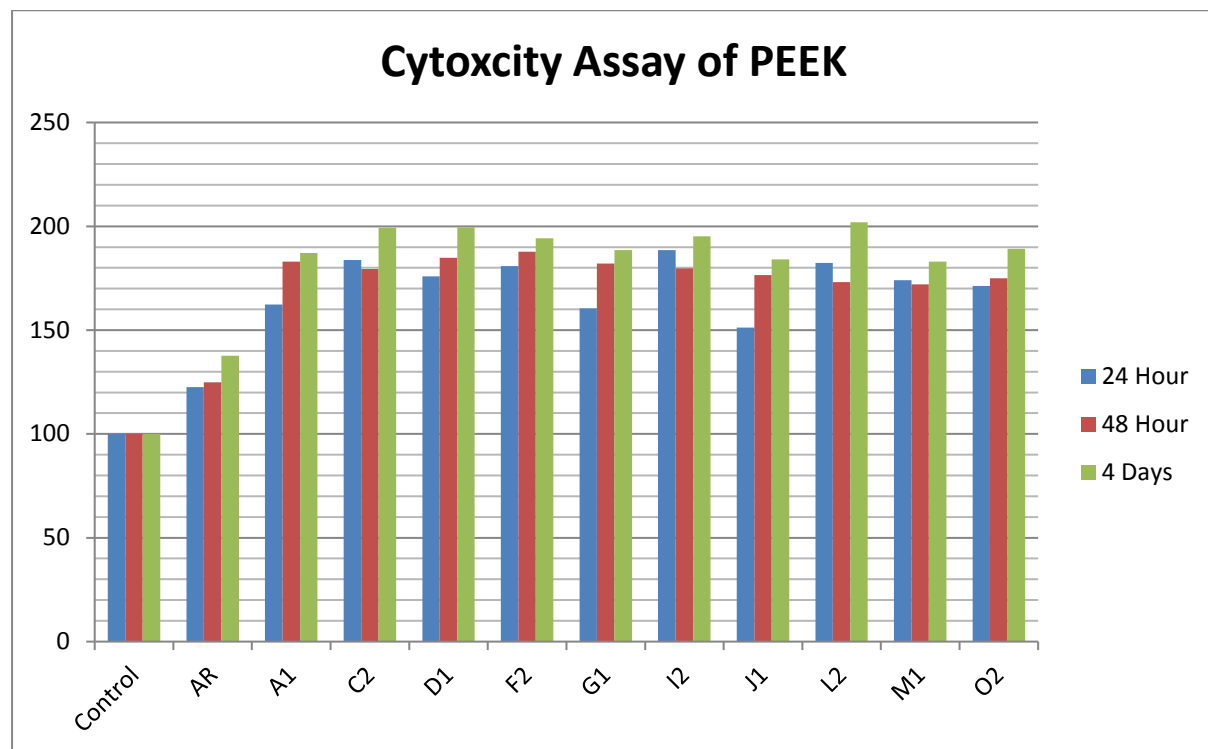


Figure 18:hMSC cell populations as shown using MTT cytotoxicity assay in the presence of PEEK +/- SE (n=11 per material)

Pairwise comparison was used to establish if there was a statistically significant difference between incubation times used within the material groups within the study. The results showed that there was a clear difference between the 24-hour incubation and both the 48 hour and the 4-day incubation with a statistically significant result being produced. While in every instance the 4-day incubation time was higher than the 48 hour incubations, when analysed, the difference was not found to be statistically significant.

3.4.3 PEEK and Nylon 6'6 Cytotoxicity Comparison

Table 13 shows that in all instances, the PEEK results were found to be higher than those exhibited in Nylon 6'6. This tallies with the significance value seen when the two are compared via independent t test where the value produced was shown to be highly significant.

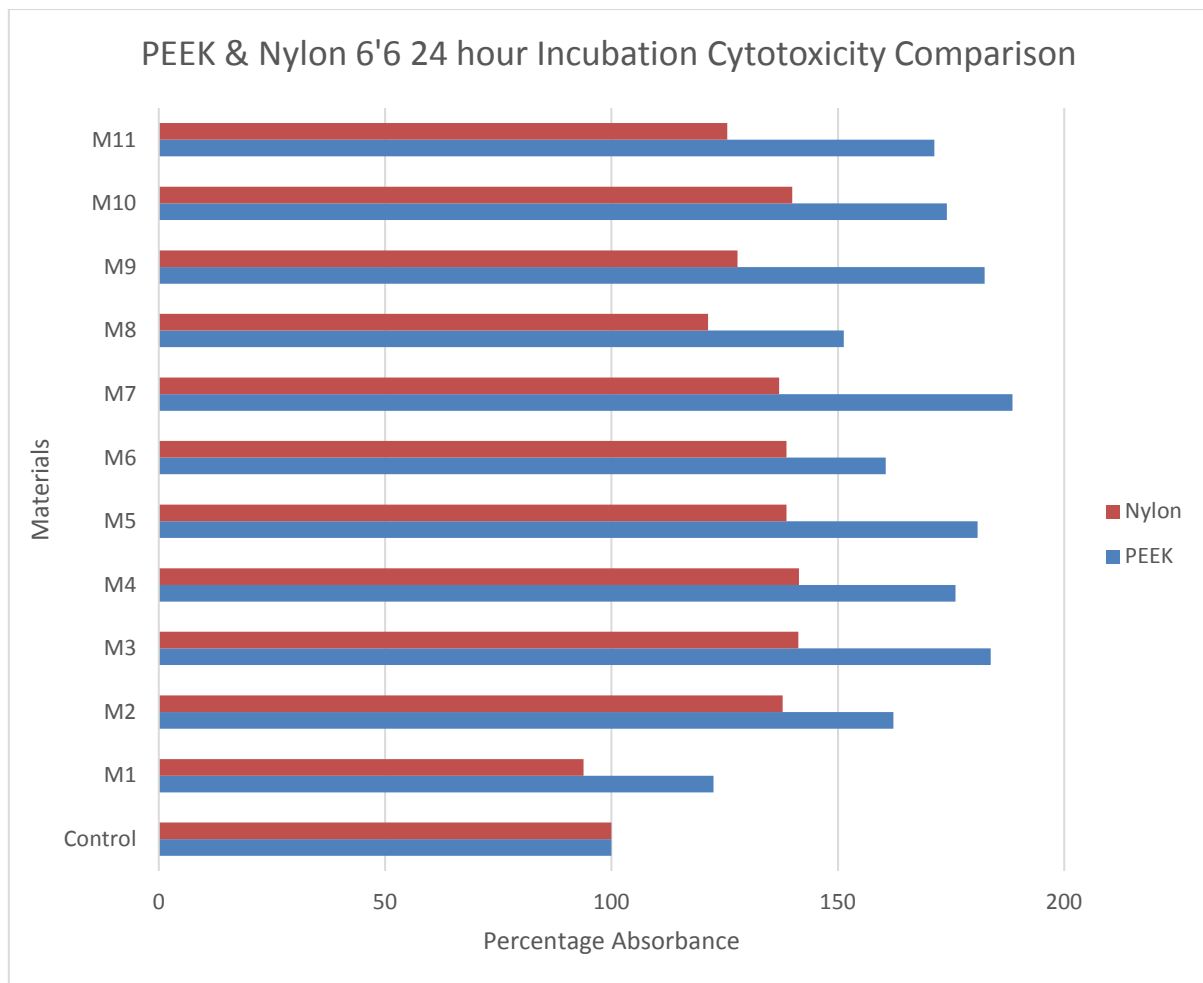


Figure 19: Comparison of the stem cell population in the different materials over a 24-hour incubation time +/- SE (n=11 per material)

The trends that were observed during the 24 hour MTT assay are once again repeated in the 48-hour incubation. While the results were higher, the value produces were shown to not be statistically significant. That being said, the values produced by the assay are higher than those previously produced.

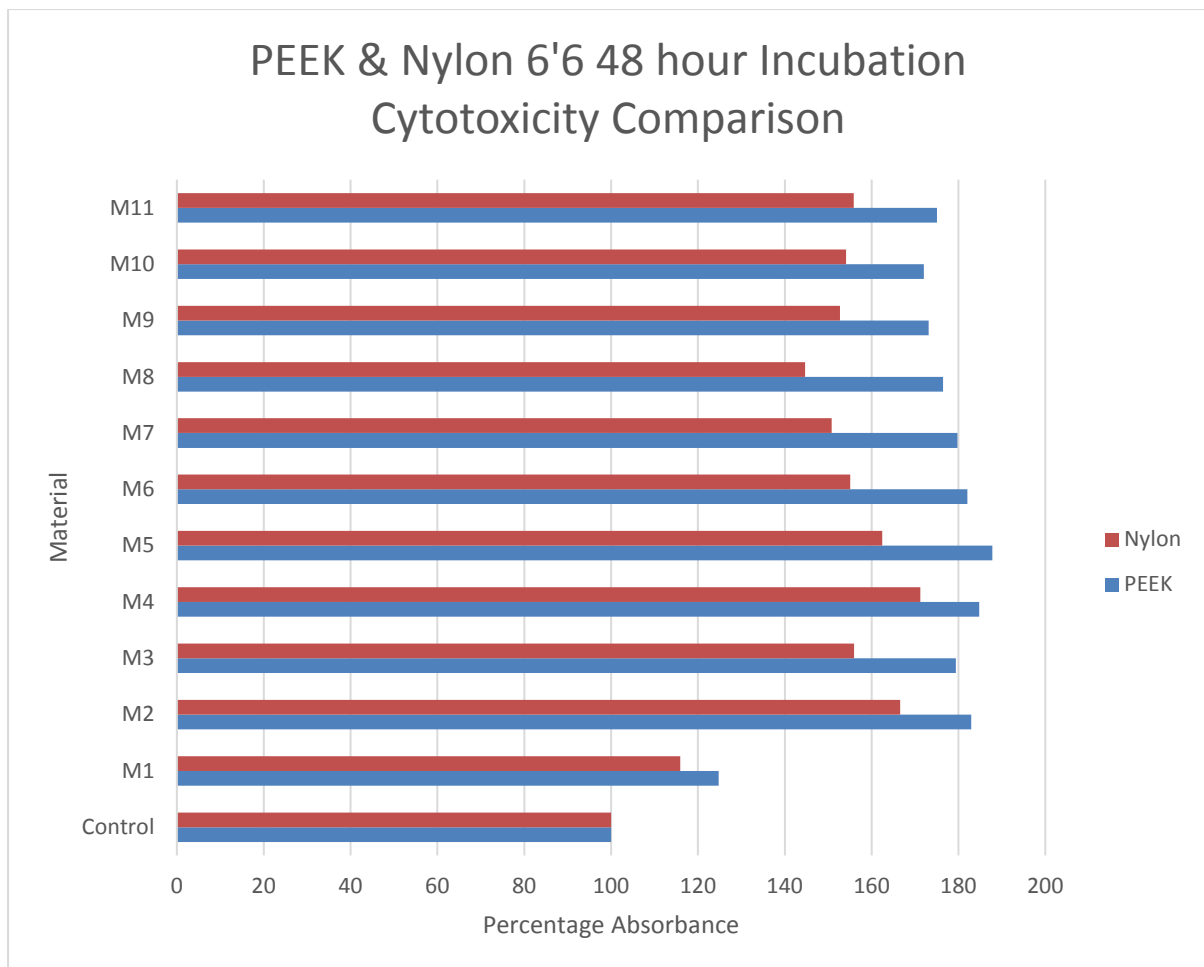


Figure 20: Comparison of the stem cell population in the different materials over a 48-hour incubation time +/- SE (n=11 per material)

The results of the 4 day MTT assay indicate that the cell population observed in the PEEK sample was much higher than the population level observed in Nylon 6'6. Despite this, the results show that there was no statistically significant difference between PEEK and Nylon 6'6, with the significance value falling far short of the required confidence level.

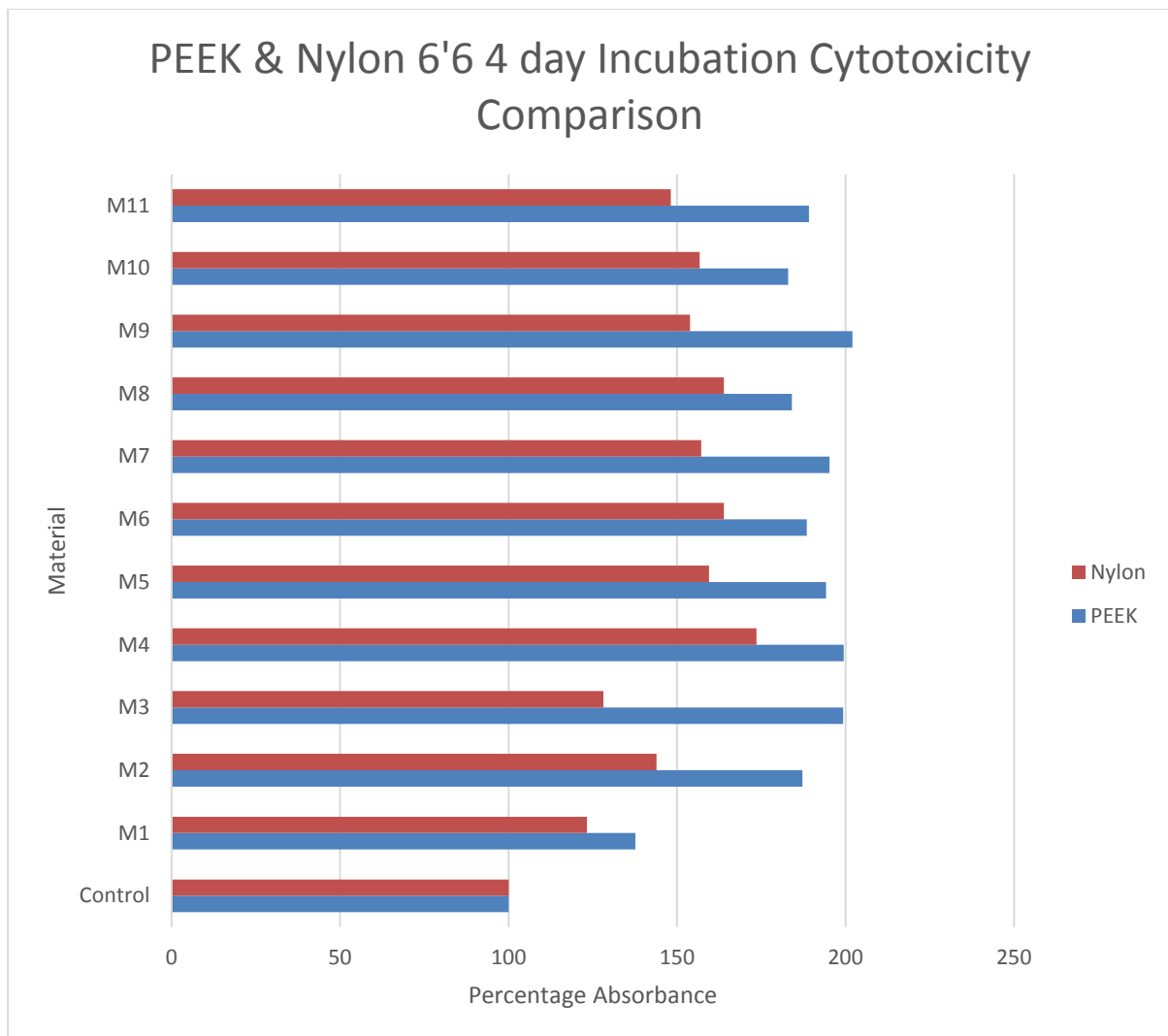


Figure 21: Comparison of the stem cell population in the presence of PEEK and Nylon 6'6 over a 4-day incubation time

3.5 Scanning Electron Microscopy

Under the SEM, a number of features are apparent in the PEEK samples used. Even on samples where no overt physical patterns were formed cell patterns can be seen on the surface. In figures 18 and 19, surface patterns can be observed, highlighted by the presence of the cells, orientated from the bottom left corner of the image towards the top right. This pattern corresponds with the pattern inscribed on the material during surface processing. This could also be related to the chemical composition of the biomaterial meaning that cells in this area grew more preferentially to other areas of the surface.

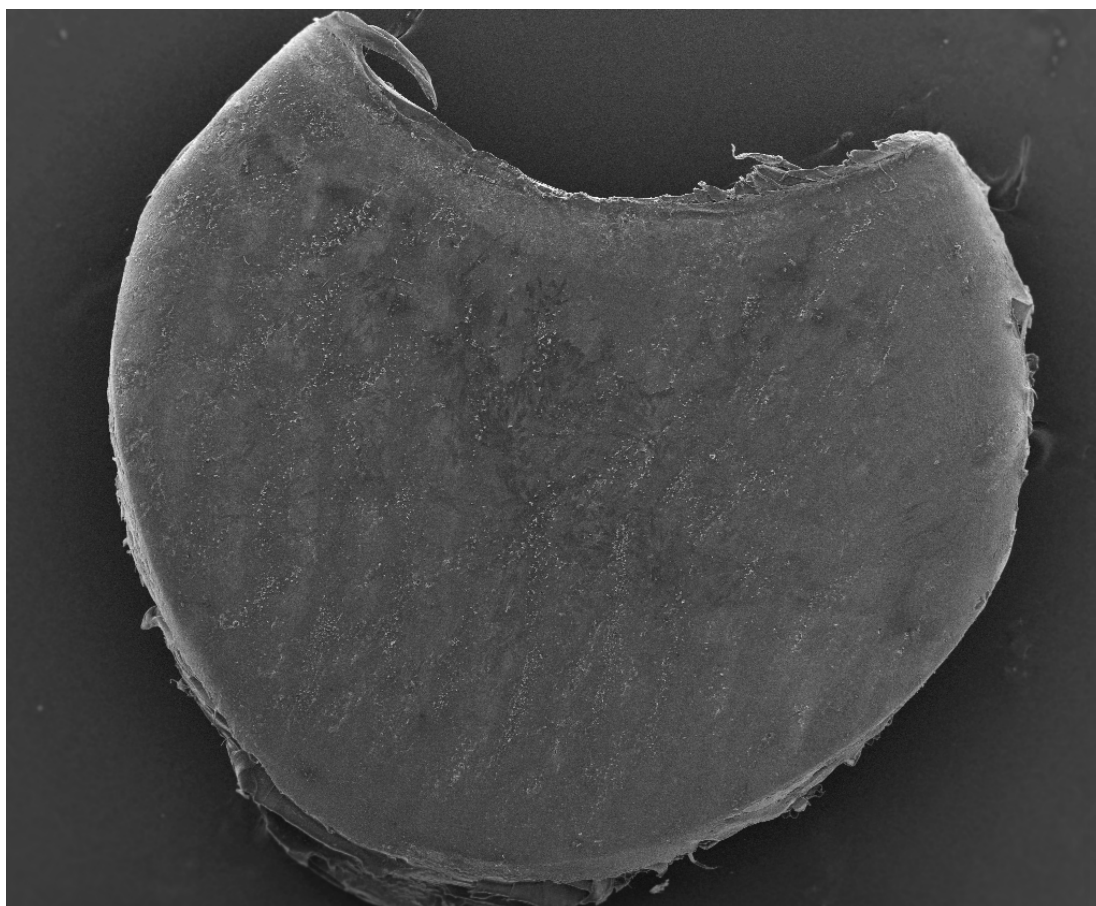


Figure 22: Magnified SEM images showing the surface of Nylon 6'6 sample A1 which was exhibited to some of the highest amounts of laser energy per square ml (x38 magnification)

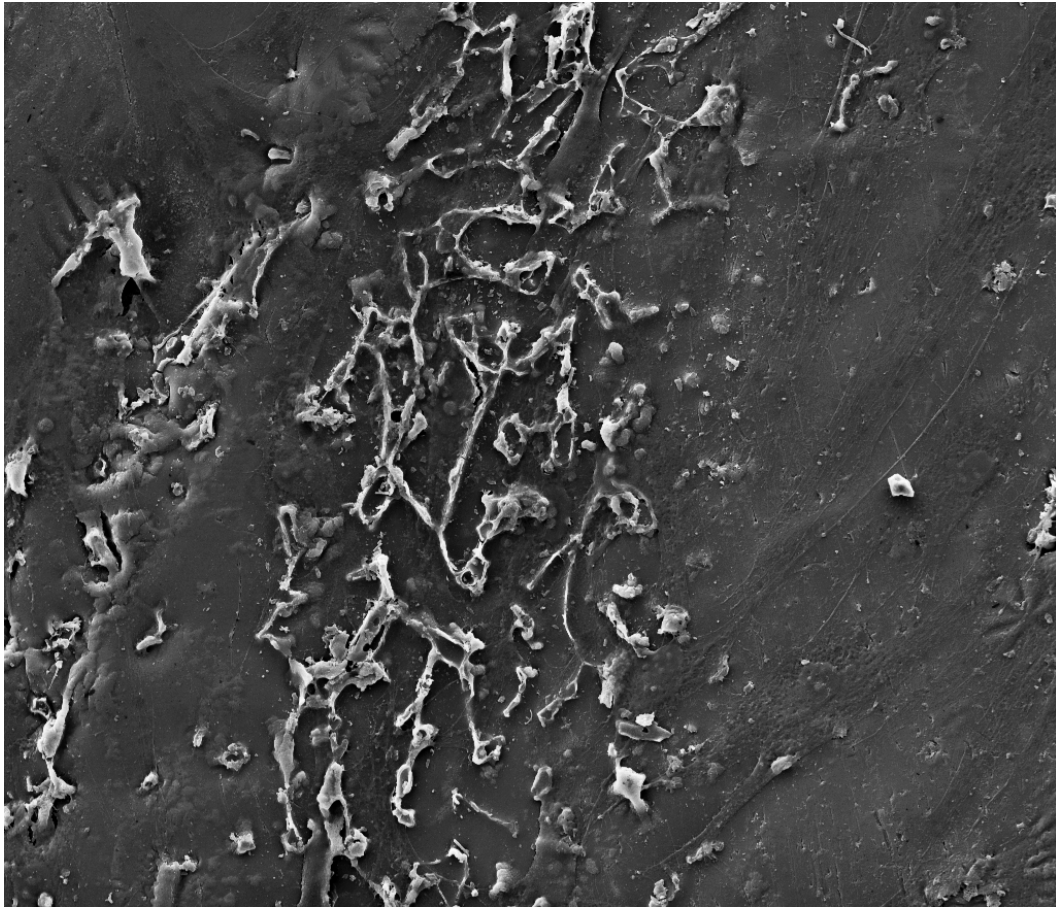


Figure 23: Magnified SEM image showing the surface of Nylon 6'6 sample A1 and the preferential growth area of hMSCs following the patter of the surface processing (x808 magnification)

A key processing issue that arose with using PEEK, when sample coupons were punched from the original processed sample it resulted in cracking and modification of the material near the edges of the coupon. This can be observed on Figure 25.

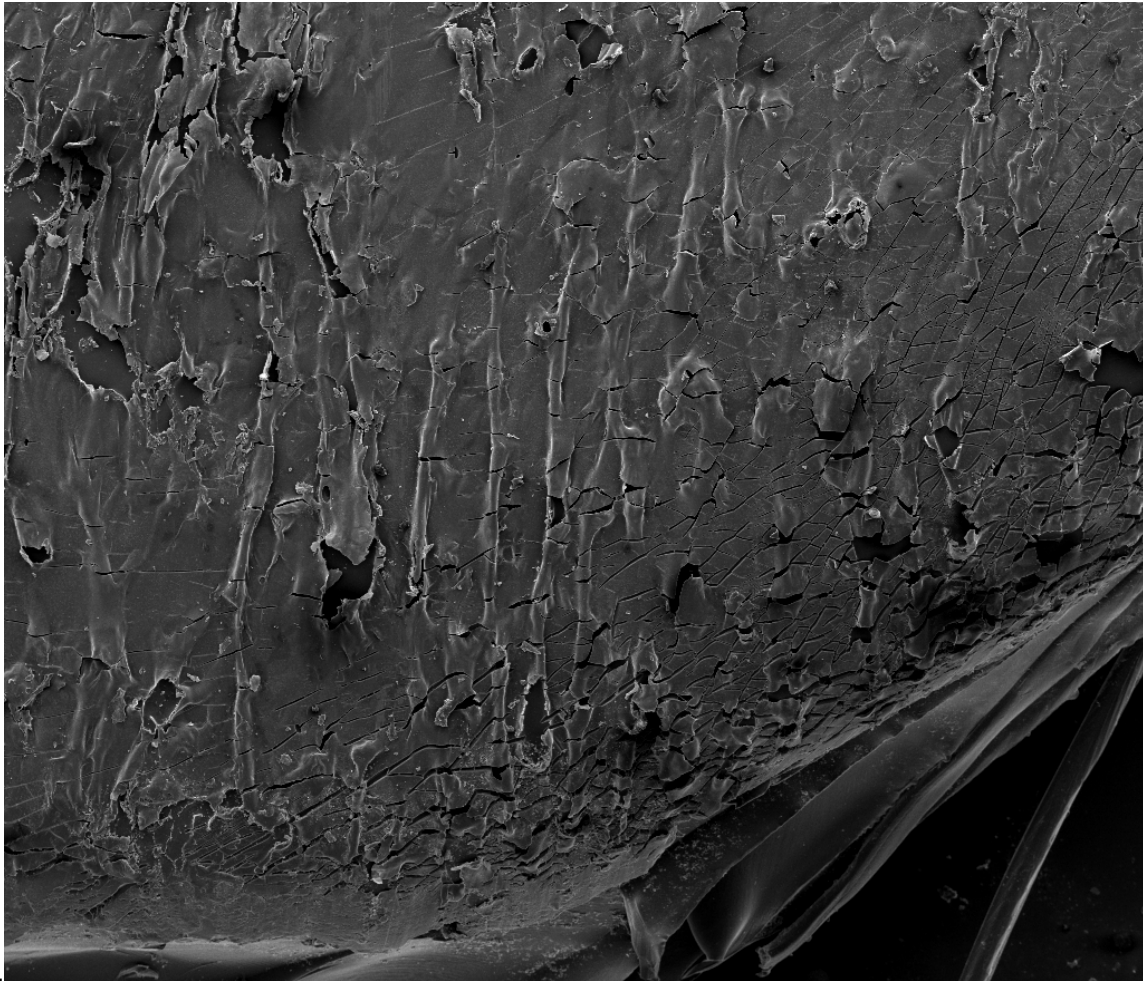


Figure 24: PEEK material cracking and surface modification as a result of the production of surface coupons from the processed sample.

3.6 Bone Morphogenic Protein 7 Assay

Figure 27 shows the average BMP-7 absorbance readings observed during the ELISA assay. The distribution of the BMP results does not correlate with the results observed during the MTT assay. The results of the crosshatched patterned surfaces seemed to be higher than the simpler line patterns, though even so there are outliers that do not conform to this trend. The highest recorded absorbance was recorded in Nylon M11. The lowest observed reading was Nylon M12. In PEEK the highest observed result was PEEK M11 and the lowest observed reading was PEEK M8. Table 16 shows the average absorbance values obtained in the BMP-7 ELISA.

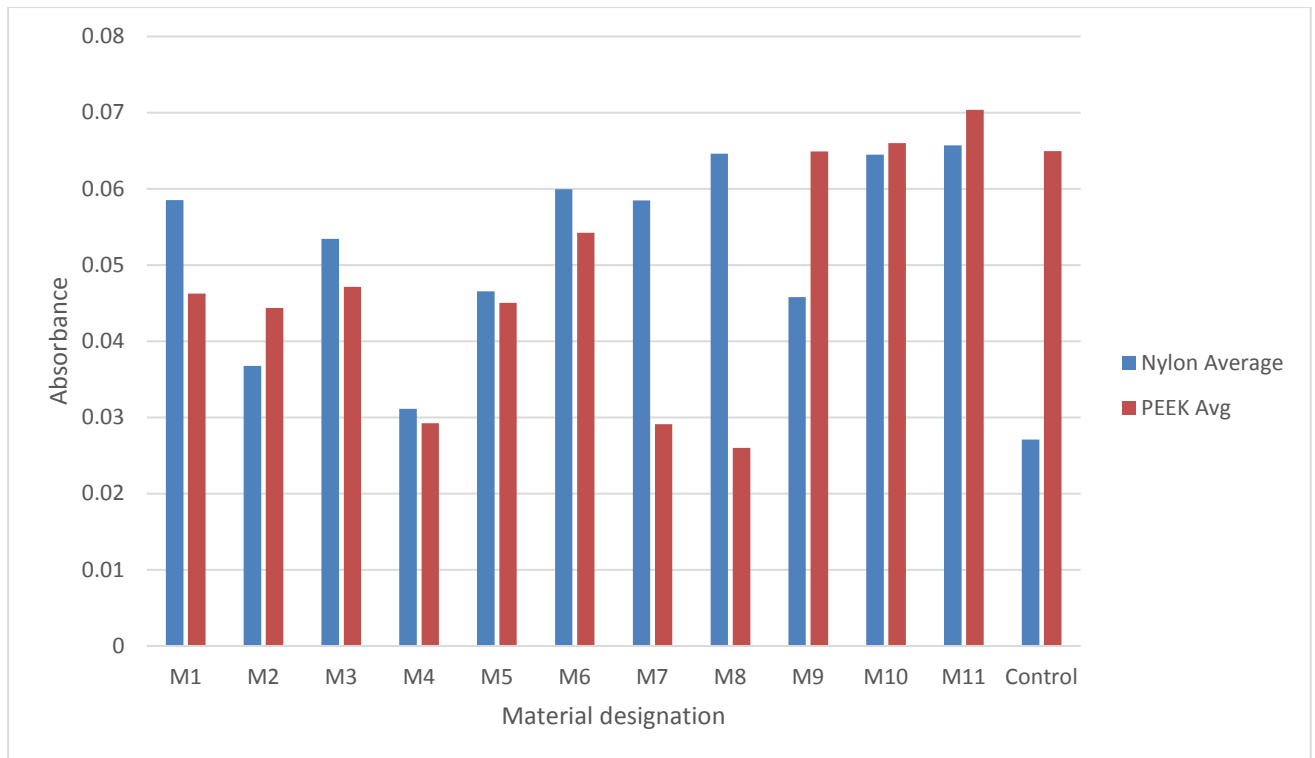


Figure 25: Average absorbance values indicating the amount of BMP-7 present in the hMSC populations cultured +/- SE (n=4).

4.0 Discussion

4.1 Growth of Human Mesenchymal Stem Cells

Human mesenchymal stem cells were used throughout the study and all were cultured under standard conditions. To prevent genetic drift and an increase in passage number, stem cells were only cultured as required. This generally involved defrosting and allowing hMSC allowing cultures to become established prior to commencement of experimental work. When cells were not required, cultures were expanded to ensure a stock was always available prior to freezing.

4.2 Wettability and Surface Roughness

The first point of interaction between biomaterials and the surrounding environment is at the material surface as such; the characterisation of the material surface and its properties are tremendously important (Arima & Iwata, 2007). An example of work in literature that exemplifies this sentiment is work by Dowling et al 2011, which investigates the effect of plasma treatment and surface roughness modification on cell adhesion and spreading. In which surface characteristic were measured using wettability analysis, the article goes on to describe these effects upon the surface of polystyrene, where water contact angle ranged from 12° to 122°. Optimum cell adhesion within the study was observed around 64°, with diminishing returns observed as both the hydrophobicity and hydrophilicity of the surfaces increased. Dowling et al 2011 then describes how rougher surfaces resulted in an increased cell adhesion between the material and the cells, however cell spreading was reduced (Dowling, et al., 2011). The hypothesis of this lends credence to this project as it shows that there is a link between modification of surface properties and effects in cell models. Beyond this it shows specifically how wettability can have an effect on cells *in-vitro* which is one of the factors recorded in this study. Work by Dowling et al 2010 work is further

corroborated by earlier work by Nishimoto et al 2008 which draws the same conclusion using MG63 osteosarcoma cells, Nishimoto et al 2008 used the same cells as the work by Dowling et al 2013 but the cells were cultured on titanium as opposed to the polystyrene (Nishimoto, et al., 2008). The works cited as well as others in literature exhibit similar results to those obtained in this study, which is encouraging. That being said there are some differences, the surfaces utilised in the work by Dowling et al 2011 were coated in siloxane, a known hydrophilic compound and because of this, while the concept is applicable in that wettability can affect cell behaviours, the exact results can't be used to give an idea of expected results as the experimental conditions vary fundamentally. In short, while the results shown in the work by Dowling et al 2011 may not be directly applicable to help predict the outcome of this study. They aid in providing proof of concept for one of the key areas of research within the study.

A number of conclusions can be drawn from the wettability results produced over the course of this study. It seemed that both materials had unique reactions to the laser processing that they were subjected to and that in some cases, distinct processing parameters elicited similar effects on the different materials. The changes in roughness seemed to have varying effects and should be considered alongside chemistry and other factors before use for biocompatibility. As has been previously noted in literature, the pattern produced by the laser itself seems to have more of an effect on contact angle than any particular roughness or chemical composition. Work by Waugh and Lawrence 2009 indicates this point perfectly where it discusses the effect of the modification of surface profile parameters on Poly Methyl Methacrylate (PMMA) and wettability properties therein. The work specifically mentions that wettability seems more to be based on pattern than any other specific parameter (Waugh & Lawrence, 2010). Another work which also discusses this point is the

Waugh et al 2010 which discusses the effect of different laser/material interaction methods upon the surface of the material. The article discusses the difference between CO₂ and F₂, and the effect there in that a number of different factors had. It is discussed that the CO₂ based system has a much wider reaching effect. In that it also effects the polar content of the material surface as well as the surface topography, namely as a result of the bond vibration and heat generated therein (Waugh, et al., 2010).

In regards of biocompatibility, wettability is one factor that can be used to categorise as a measure of biocompatibility. It has been shown repeatedly in literature that the wettability has an effect on cell adhesion. This is highlighted and discussed in work by Bacakova et al 2011 which states that an optimum wetting angle exists for an individual surface and that this can be used as a guide line for cellular response. The article discusses how the moderately hydrophilic surfaces are optimal for cell adhesion and that this is compounded in the presence of positively charged substrates (Bacakova, et al., 2011). This is caused by the adsorption of particular proteins that mediate the cell adhesion process, resulting in specific amino acid sequences being made available to cell adhesion receptors. When a material surface is too hydrophilic different sequences are exposed, or bind very weakly hampering cell adhesion. When a material is too hydrophobic the proteins adsorb in an inactive form, having the same effect (Bacakova, et al., 2011). This work lends some more credence to the underlying principles that this project that optimising material wettability has a beneficial effect on cellular behaviour. That being said, more work is required as optimal parameters for this material do not exist.

In this manner, wettability can be used as a measure of biocompatibility. While this is a qualitative concept, factors such as wettability can be used to form a picture of the

materials biocompatibility. Wettability can also be used as a predictor of cellular response and proliferation rate (Cicuendez, et al., 2012). That being said however, more research may allow for this to become a more quantitative measure.

4.3 Surface Chemical Analysis

It has been reported in literature for a number of years that the chemical composition of the surface of a biomaterial has an effect upon biocompatibility and differentiation (Kingshott, et al., 2011). Work by Chan et al 2013 demonstrated this point well when the article showed that the reduction of the nickel ratio in titanium alloys was beneficial to the growth of hMSCs (Chan, et al., 2013). Work by Yao et al 2014 discusses the manner in which Nickel and Cobalt are able to affect the process of self-renewal in embryonic stem cells. The article describes the mechanism by which the presence of Nickel and Cobalt by modulating the cell transcription factor OCT-4 (Yao, et al., 2014). This article demonstrates a consideration that is key when modifying and selecting biomaterials, does the material have the potential to have a negative effect on cell growth. That being said, even gold standard materials such as Titanium can have similar effects. Work by Tian et al 2011 follows in a similar theme, which discussed how different ratios of TiO to TiO₂ can result in the exposure of cells to in varying levels of Ni atoms. This is due to how the material surface forms under different material compositions resulting in the exposure of Ni atoms to the external environment. The article states that it is beneficial for the growth of cells to have as homogenous a surface as possible to reduce the potential for Ni atom release from NiTi alloys (Tian, et al., 2011). This does highlight a potential parameter to be controlled during material modification. Molecular compositions can have a significant effect on the dynamics of cell growth i.e. growth rate, genomic and proteomic expression among others. That being said cytotoxicity could also be a beneficial trait

depending on the desired application. What is deemed as beneficial or not is dependant entirely on the desired function of the material or the desired characteristics.

Modification of surfaces chemistries has been shown to be beneficial to cell growth in both metallic biomaterials and polymeric biomaterials. Beyond this however, an increase in oxygen content via specific molecular species was also shown to be beneficial. This was elicited by reducing the titanium to oxygen ratio via the use of laser surface treatment to replace titanium and nickel with atmospheric gasses, these generally consisted of oxygen and nitrogen species with trace levels of carbon (Chan, et al., 2013). A similar result was elicited in the polymeric materials utilised in this project. The species of atoms that are added to a material surface when it is heated is directly comparable to the materials present in the atmosphere, as a result, nitrogen was the most commonly added element. This has been shown in literature on a number of occasions to have a beneficial effect on a variety of cellular processes *in-vivo* (Carrero-Sanchez, et al., 2006). That being said, the efficacy of this method of modifying is dependent on specific processing parameters, as a result it could be beneficial to process materials purely in the presence of atmospheric conditions present, more research is required into this specific parameter.

An article that discusses this potential in detail is work by Wan et al 2010 which discusses the biocompatibility of nickel-free high nitrogen stainless steel (NHS) and the effect there in on blood compatibility. The article discusses how NHS was shown to have a higher biocompatibility when compared to a more conventional austenitic stainless steel. The article discusses how the coagulation time was lower in the NHS and that the NHS has a lower contact angle at every stage of testing (Wan, et al., 2010). Taking into account the benefit of the presence of oxygen on cell growth, it

could be hypothesised that increasing the proportion of oxygen within the atmosphere could produce an improved cellular response. Work by Wan et al 2010 how is that different factors can be combined together to provide a composite picture of biocompatibility. That being said, wettability and chemical composition are heavily interlinked, as wettability is in part determined by the chemical composition at the material surface as well as topographical factors (Rupp, et al., 2014).

4.4 Surface Topographical Analysis

Over the course of this project, a number of different methods of surface visualisation were evaluated for use, the key options that were considered were White Light Interferometry (WLI) and 3D Profilometry (3DP). WLI produces a value based upon surface profile roughness (Ra) value and also produces a surface visualisation, with a number of image processing options available. The spectrum displaced upon the material was used to give an indicator of height while still in 2D. Whereas the 3D profilometer produced an area roughness parameter (Sa) value as well as surface visualisation image.

The 3DP was deemed to be the better option for a number of reasons. The main one being that the Sa value produced by the 3DP is an average of the area measured by the 3DP whereas the Ra value produced by the WLI is a linear average of the entire surface roughness. While both are comparable, Sa is preferred in literature (Gadelmawla, et al., 2002).

Work by Ghosh et al 2013 compared the use of both SEM stereoscopic imaging and atomic force microscopy (AFM) on bovine cartilage. The article described how SEM stereoscopic imaging and AFM were both utilised to produce a roughness value for the same material. The study concluded that both forms of surface roughness provided

adequate data to draw conclusions regarding roughness. However, the article highlighted that AFM took a longer period of time to produce an accurate scan of the bovine cartilage used in this study took approximately 12 hours. (Ghosh, et al., 2013). This above work highlights the potential utility in the scanning of surface roughness and as such could be a potential avenue of future research.

Surface topography has an effect on biocompatibility in a number of different ways. An article by Waugh and Lawrence 2010 illustrated one way in which surface topography can have an effect upon wettability. It discusses how the spacing between the lines within the pattern can result in different wetting profiles (Waugh & Lawrence, 2010). This is generally used a measure of how well stem cells would adhere to the material surface and how well they would spread. Work by Chan et al 2013 shows how cells conform to the surface on which they are found and as a result patterns can result in impaired adhesion characteristics (Chan, et al., 2013).

In terms of biocompatibility, topography can be used to affect a number of changes, however it generally seems to be marginally less important than the chemical composition of the surface (Chan, et al., 2013). That being said, surface topography can contribute to towards creating a bias towards certain cell lineages or fates. Natural surfaces have been shown to have a topography that reduces the survival rate among cells and bacteria (Ivanova, et al., 2012). Surface topography is important to determining biocompatibility but less so than chemical composition (Chan, et al., 2013). However, as biocompatibility as a whole is a purely qualitative measure at the current time, this may only be true in this instance.

4.5 Cytotoxicity Testing

The results of the MTT were key to most of the hypothesis within this study. The results showed that in both cases the biggest difference in mean was between the time course 1 (24 hours) and time course 2 (48 hours) and there was not a large amount of difference between the 48 hour and the 4 day results. The main difference between the differences between time course 2 and time course 3 (4 days). This is due in part to the time that the cells spend in the lag phase that occurs while cells attach to the material surface and re-establish their standard cellular metabolic processes.

All incubation times, in both materials were found to be significantly different than the 24-hour group, however the differences between the 48 hour and the 4-day group were shown to not be statistically significant, though there was a positive increase in PEEK between 48 hours and 4 days. The results show that generally the 4-day incubation for PEEK was higher than that of Nylon 6'6 which is also shown on table 17. The mean absorbance for PEEK after 4 days was 187.247, this indicated that the absorbance of the samples grown on PEEK were 87.2% higher on than the control samples over the same period of time producing significant results. Nylon was shown to be almost 52% higher than the control sample over 4 days. These results indicate that laser surface treatment has a positive effect on cell proliferation when compared to the unmodified material surface across all incubation times.

4.6 BMP-7 Protein Expression

The ELISA assay results showed that there was no correlation between the absorbance results observed in the MTT assay. This is due to the fact that the surfaces produced did not provide any cues for osteogenic differentiation and as a result the cell population remained heterogeneous and there was no marked increase in the levels of BMP-7 expressed. That being said, this does give clear

avenues for future research, and literature does show that it is possible to induce selective differentiation without the use of osteogenic differentiation leading media. Work by Hu et al 2011 discusses the interactions of C2C12 myoblasts and human bone marrow stem cells (hMBSC's) with silk-tropoelastin biomaterials and the capacity for differentiation therein. The article states that a low surface roughness and high level of material stiffness was favoured by C2C12 cells with a lesser importance being placed upon micro/nano scale surface patterning. Where as in HMBSC's, a higher surface roughness and stronger level of micro/nano scale surface patterns were favoured by hMBSC's. These parameters were shown to produce increased levels of proliferation as well as osteogenic differentiation (Hu, et al., 2011). The article highlights the notion that it is possible for material properties alone to be able to induce stem cell differentiation independent of the presence of differentiation leading media. That being said it could be possible for the article to be taken further by comparing the results seen within the study and the results seen in a similar study in the presence of differentiation media. Work by Watari et al 2011 describes the use of submicron scale topographically patterned ridges and grooves and their role in the modulation of osteogenic differentiation. The article showed that 400nm pitch materials enhanced extracellular calcium deposition as well as exhibiting markers of osteogenic differentiation. The article also investigates the effect of the presence of differentiation media on hMSC's when grown upon 400nm pitch materials. The results showed that the osteoinductive properties of the material were enhanced by the presence of the osteoinductive media (Watari, et al., 2012). The article is positive one for this study in that it highlights the ability for materials to be what is considered osteoinductive and also discusses the effect of introducing cells cultured on these materials to osteoinductive media. The article also discusses

how the presence of the osteoinductive media worked in a complementary manner with the osteoinductive cues provided by the 400nm planar surface. This resulted in a greater result than would have been otherwise achieved. This article further lends credence to the concept that topographical cues can be used to elicit a differentiation leading response. While in this instance this effect was not produced, a response was still provoked, it is possible that culturing HMSCs in osteoinductive media on laser treated polymer materials would result create a differentiatinal bias in the increased hMSC population observed with polymeric materials.

5.0 Conclusion

In conclusion, the rate of proliferation of hMSCs on both PEEK and Nylon 6'6 was much higher when the cells were cultured in contact with biomaterials that had undergone surface processing compared to both the unprocessed samples and the control. A highly statistically significant difference was observed in rate of hMSC growth when incubated for 24 hours compared to when hMSCs were incubated for 48 hours and 4 days. This was the case in both materials that were utilised in this study. However, the observed difference between the rates of hMSC growth between 48 hours of incubation and 4 days was shown to not be statistically significant. Despite this however, the rate of proliferation observed as shown by cytotoxicity testing was shown to much higher than the unmodified material samples which were in themselves much higher than the samples grown without the biomaterials.

There was no observed correlation between rate of cell growth on processed material surfaces and expression of BMP-7 in culture. This is most likely due to the heterogeneous genetic composition of the cell population used. Any potential increases seen were concurrent with the rate of population growth, indicating no increase in rate of expression. That being said, using material properties to influence lineage fate has been shown to be conceptually viable in literature, and warrants further investigation.

The results indicate that the application of laser surface processing to polymeric biomaterials had a positive effect on the rate of cell proliferation and expansion in both materials. This was achieved through modification of the topography and surface roughness and by modifying the chemical composition of the material surface, in this case primarily by modifying the concentrations of oxygen and nitrogen present in

comparison to the unmodified samples. The results show that the biocompatibility of polymeric biomaterials is further enhanced by the application of laser processing.

6.0 Avenues of Future Study

It is a commonly agreed upon fact that a myriad of cues from the microenvironment surrounding target cells affect both intra and extracellular mechanisms and behaviours (Kshitiz, et al., 2013) Ranging from increased proliferation or protein production to induced apoptosis as a result of genetic errors as is seen in cancer prevention (Gribova, et al., 2011) (Pecorino, 2012).

In terms of future research and potential study, biomaterial modification is an area that is still in the vast majority open. A large number of research avenues, in some cases the majority of avenues are currently unexploited or only the most preliminary work is currently published. A pervading theme among current research efforts is aimed at producing processing parameters that elicit an improved biocompatibility. This may result in a number of different parameter sets for individual materials but general themes lead towards the augmentation of natural human body processes.

6.1 Potential Alternative Materials

An overwhelming majority of literature indicates that the level of cell adhesion observed can be enhanced in the presence of a rougher material surface as opposed to a smoother one, as described in work by Faeda et al 2009 and Kakura et al 2014, despite being on different materials demonstrates this point (Faeda, et al., 2009) (Kakura, et al., 2014). Work by Rosales-Leal et al 2010 corroborates this, the work investigated the effect of various different methods of processing titanium material on the culture of osteoblast like cells. It indicates that there is a correlation between increased surface roughness and increased cell adhesion. However, the article also states that this occurred at the expense of proliferation in both aluminium blasted and aluminium blasted and acid etched samples after 24 hours. After 48 hours however, cell proliferation was shown to occur at an improved rate above what was observed

on untreated materials in both acid etched and blasted and etched samples (Rosales-Leal, et al., 2010). One particular mechanism through which the roughness of the surface could affect the rate of cell proliferation is through the cells spreading over the surface to obtain an adequate level of adhesion which would also explain the lower levels of spreading compared to none treated surfaces. Work by Pattanaik et al 2012 discusses this very point, not just in regard to surface roughness but in regards to modification of surface modification as well (Pattanaik, et al., 2012).

A great many avenues of research within material science especially those relating to the use of materials in biological models are uncultivated. The vast majority of research avenues and potential applications are still unexplored or only the most basic preliminary work has been performed. One major exception of work relating to body-titanium interactions and dynamics, though this is due to its wide spread use in both medical and dental implantation as opposed to any specific reason.

One area that has drawn a large amount of interest is investigation as to what alternatives exist for materials such as titanium for instances cases where alternatives may be medically preferable or cheaper. Work published by Riverio et al 2013 mentions that PEEK is a good alternative to a number of materials currently considered in implantation such as gold standards such as titanium (Riveiro, et al., 2013). A large number of research avenues are currently unexplored in terms of material use and most of those that have been examined have been done in the most basic of terms, the number of avenues for potential materials is huge.

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Appendices

Material	Manufacturer
1.2% glutaraldehyde in water (1.2ml of 10% glutaraldehyde in 10ml of water)	Fischer Scientific, UK
3D Profilometer	Tallysurf Series 2, Taylor Hobson
Absolute Ethanol 70% ethanol 30% water	Fischer Scientific, UK
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich
Generic Freezer (235.15k)	Generic
Goniometer	Ramè-Hart Instrument Co.
Human BMP-7 ELISA Kit	Sigma Aldrich
Human Mesenchymal Stem Cell	Stem Cell Bank, Japan
Incubator (MCO-170AIC)	Panasonic
Beaker (3 litres)	Sigma-Aldrich
Liquid Nitrogen Container (-208.15°C)	Thermo-Scientific
Michigan Cancer Foundation – 7 (ECACC)	Michigan Cancer Foundation
Microbiology Safety Cabinet	BioMAT-2
Gilson Micropipettes	Gilson Inc
Gilson Micropipette tips (E1013, E1012, E1010)	Gilson Inc

MicroPublisher Camera (MicroPublisher 3.3 RTV)	QImaging Corp, Canada
Mr. Frosty™ Freezing Container	Thermo Scientific
Nunc CryoTubes	Sigma-Aldrich
Optical Microscope (Olympus, BX51; Nikon Eclipse TS100)	Nikon
Phosphate buffered saline (PBS) at pH 7.4 (9.88g PBS powder in 1 litre deionised water)	Fischer Bioreagents, UK
Scanning Electron Microscope (JCM-5000)	(JEOL Ltd, Japan)
Stop clocks	
Sterile Gilson Micropipettes	Gilson Inc
Sterile Gilson Micropipette tips (E1013, E1012, E1010)	Gilson Inc
Ultra-Low Temperature Freezer (HEF-U570) (-80 °C)	New Brunswick Scientific
Water bath (OLS 200)	Grant

Material Designation	Power (%/25W)	Spacing (µm)	Speed (mm/s)
AR-M	0%	0	0
M1	25%	50	250
M2	25%	150	500
M3	50%	50	250
M4	50%	150	500

M5	12.50%	50	250
M6	12.50%	150	500
M7	25%	50	250
M8	25%	150	500
M9	12.50%	50	250
M10	12.50%	150	500

Table 11: Sample Material Processing Key

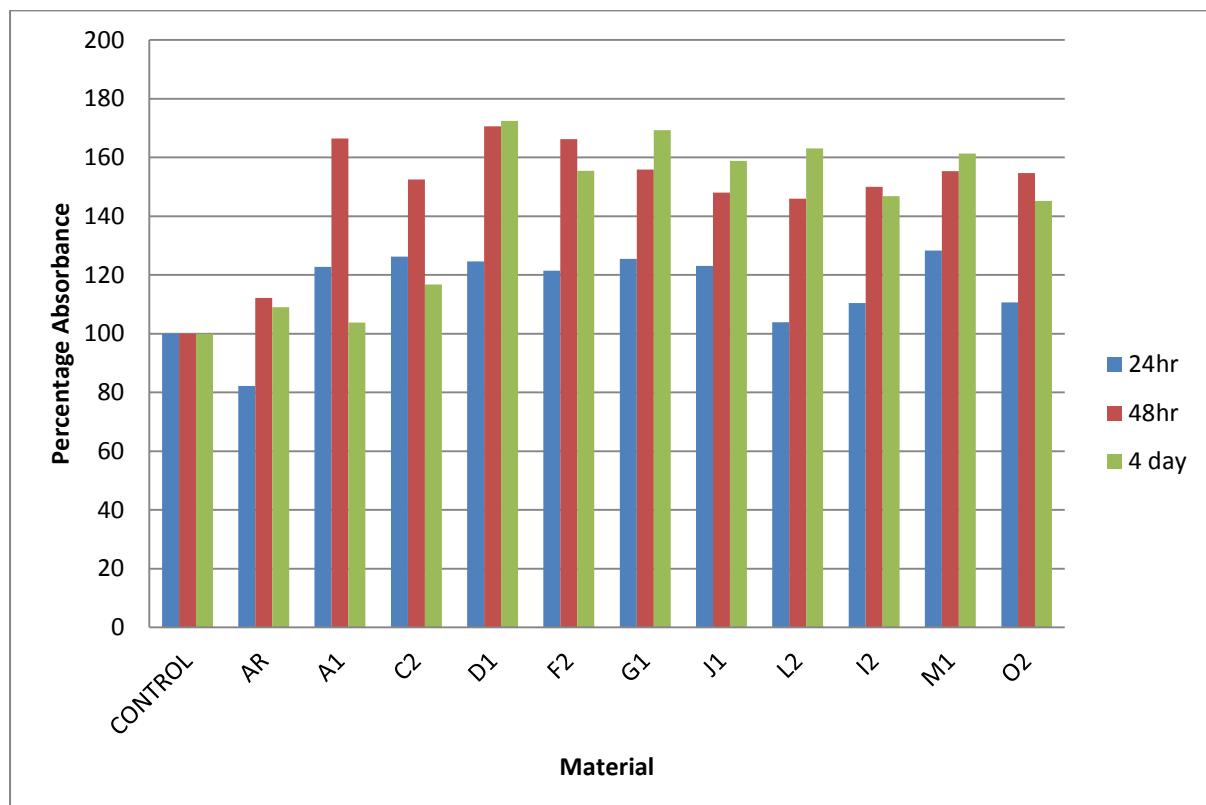


Table 12: Averaged MTT Results for the first round of Nylon Cytotoxicity Assays

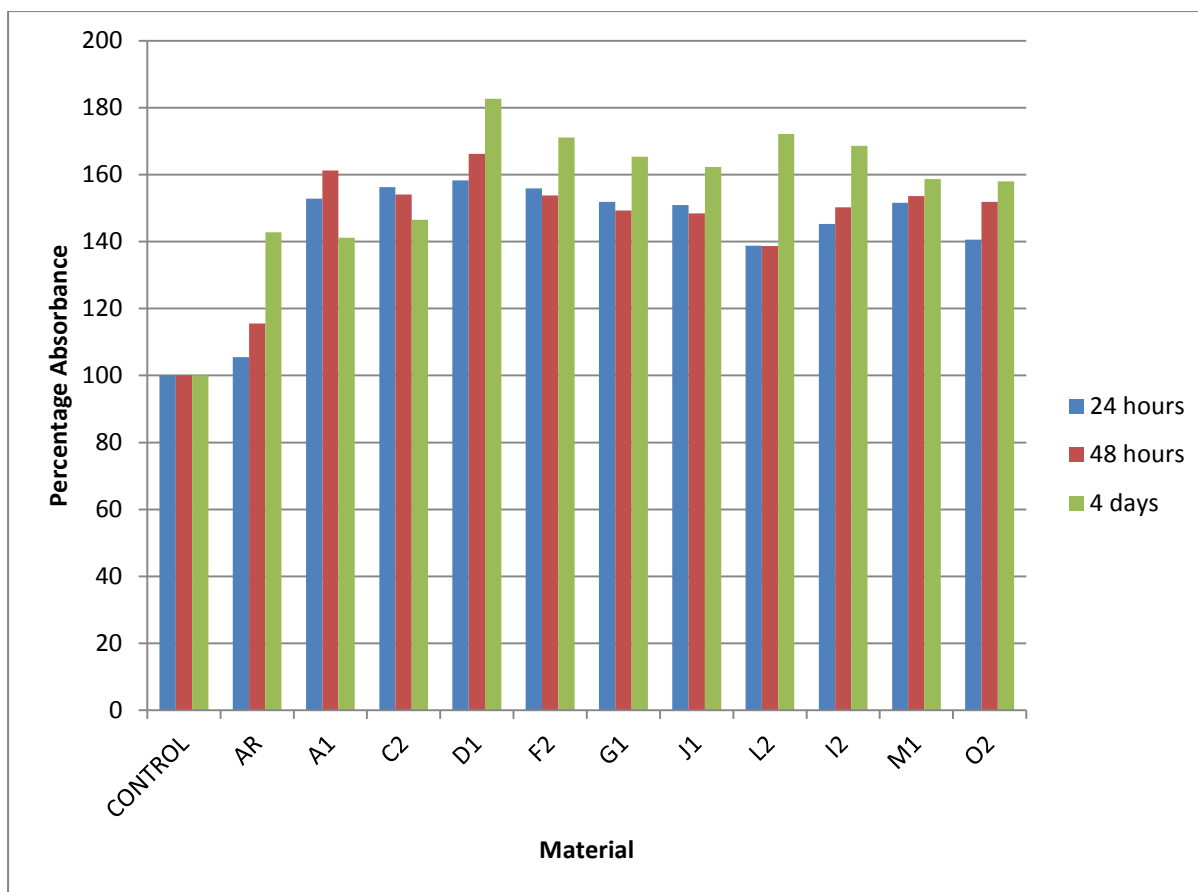


Table 13: Averaged MTT Results for the Second Round of Nylon Cytotoxicity Assays

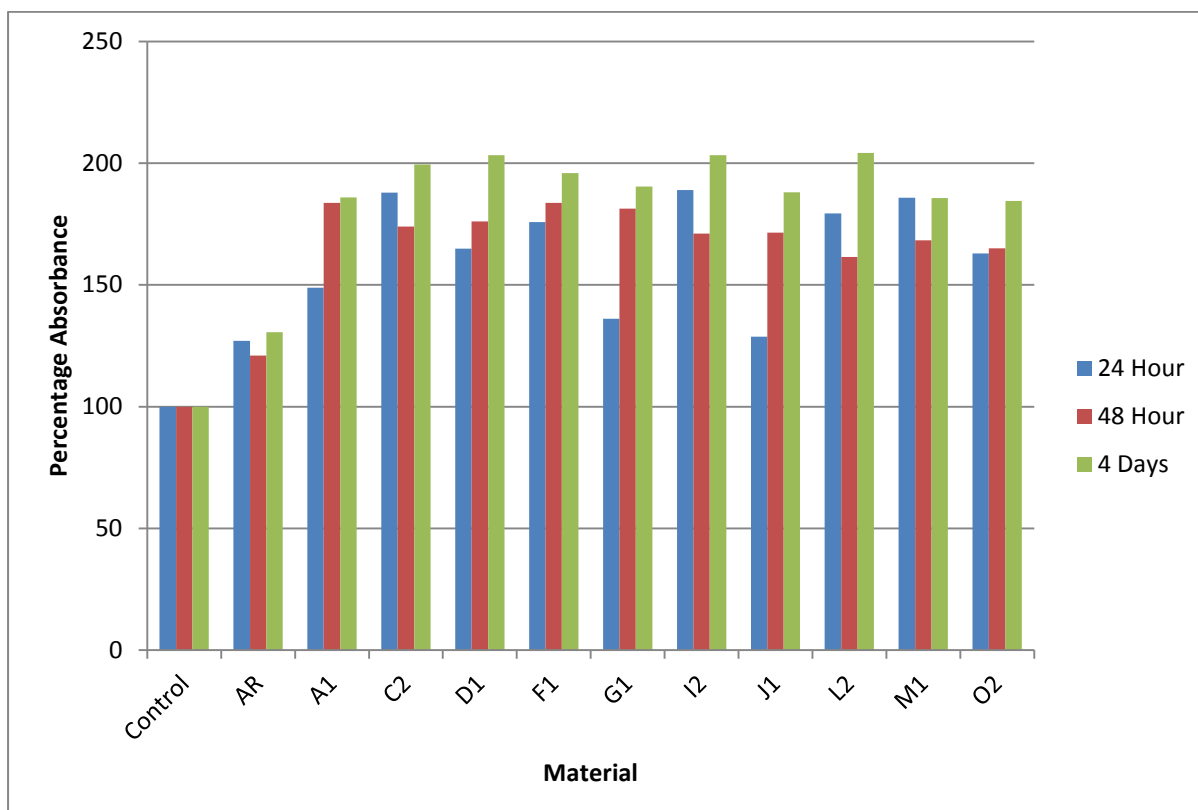
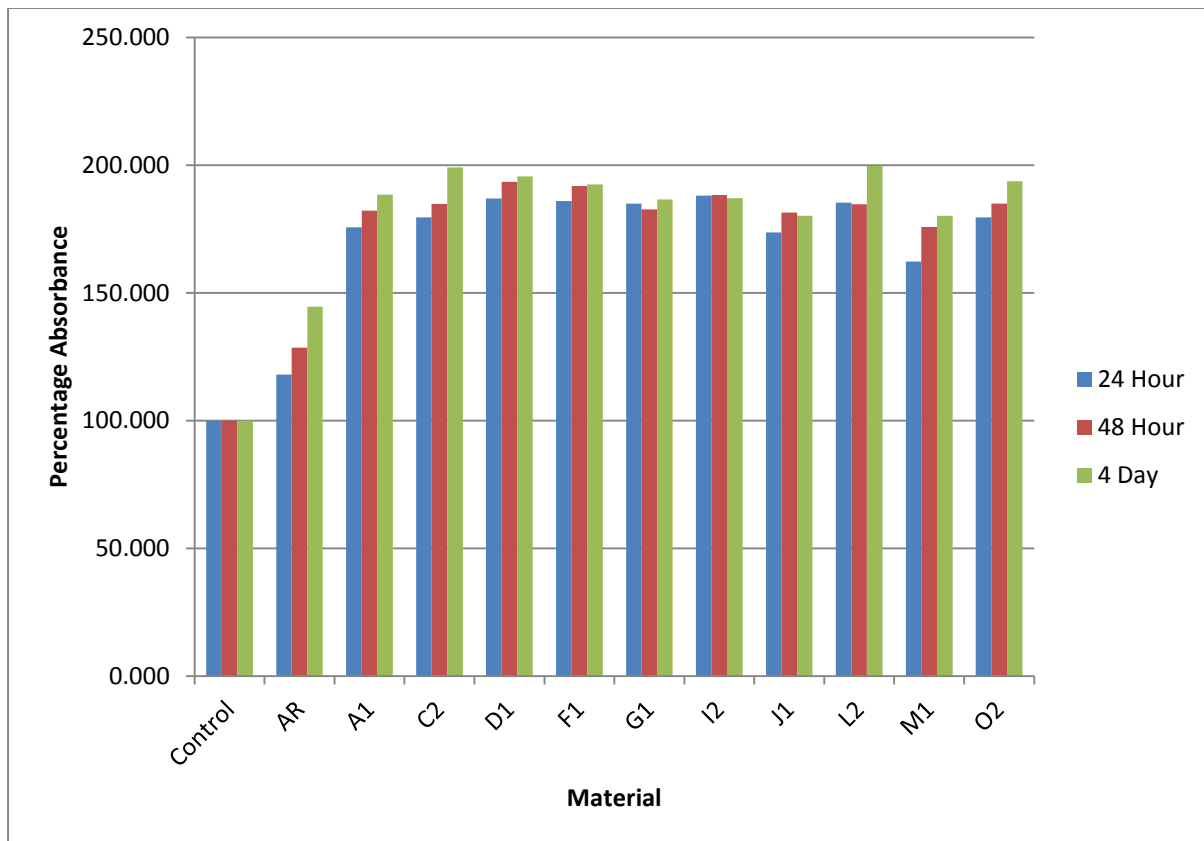


Table 14: Averaged MTT Results for the first round of PEEK Cytotoxicity Assays**Table 15: Averaged MTT Results for the Second Round of PEEK Cytotoxicity Assays**

(I) Time	(J) Time	Mean Difference (I-J)	Std. Error	Sig. ^b	95% Confidence Interval for Difference	
					Lower Bound	Upper Bound
1	2	-13.941*	2.066	.000	-19.340	-8.543
	3	-19.759*	2.502	.000	-26.295	-13.222
2	1	13.941*	2.066	.000	8.543	19.340
	3	-5.817	2.338	.065	-11.925	.291
3	1	19.759*	2.502	.000	13.222	26.295
	2	5.817	2.338	.065	-.291	11.925

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

Table 16: A Pairwise Comparison evaluating the statistical significance between the three different incubation times used in both materials.

Material	PEEK	NYLON
M1	10.3	14.7
M2	7.98	1.56
M3	24.8	24.8
M4	59.8	4.62
M5	34.6	1.69
M6	5.17	4.73
M7	1.43	3.31
M8	18.4	43.2
M9	9.44	3.85
M10	10.1	22
AR	42.43	1.77

Table 17: Surface Roughness of PEEK and Nylon found using 3D Profilometry

PEEK	Contact Angle	Nylon 6'6	Contact Angle
AR-M	70.24	AR-M	53.54
P1	125.03	P1	40.43
P2	84.65	P2	77.74
P3	132.83	P3	00.00
P4	78.89	P4	55.00
P5	72.58	P5	52.70
P6	61.88	P6	60.89
P7	88.14	P7	57.97
P8	96.37	P8	65.48
P9	69.46	P9	43.54
P10	68.91	P10	62.01

Table 18: Averaged contact angles for both PEEK and Nylon 6'6

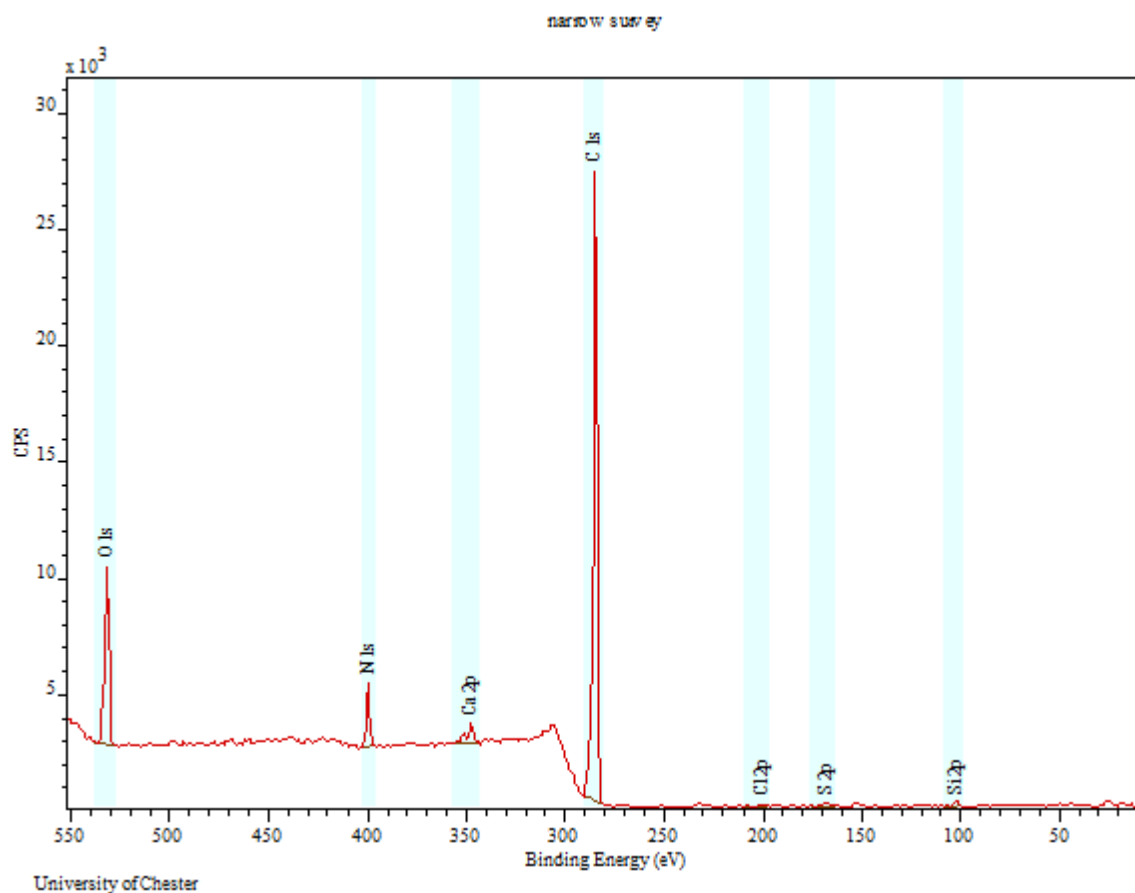


Table 19: A graph of the elemental composition of as received PEEK

Name	Position	FWHM	Area / (T*MFP)	%At
				Conc
C 1s	284.78	1.885	60269.66	82.47
O 1s	531.78	2.607	23803.99	11.12
N 1s	399.78	1.917	6002.48	4.56
Ca 2p	347.28	2.17	3352.2	0.9
Si 2p	102.28	2.083	348.6	0.58
S 2p	168.28	2.523	328.54	0.27
Cl 2p	200.28	1.744	153.68	0.09

Table 20: The full elemental composition of as received Nylon

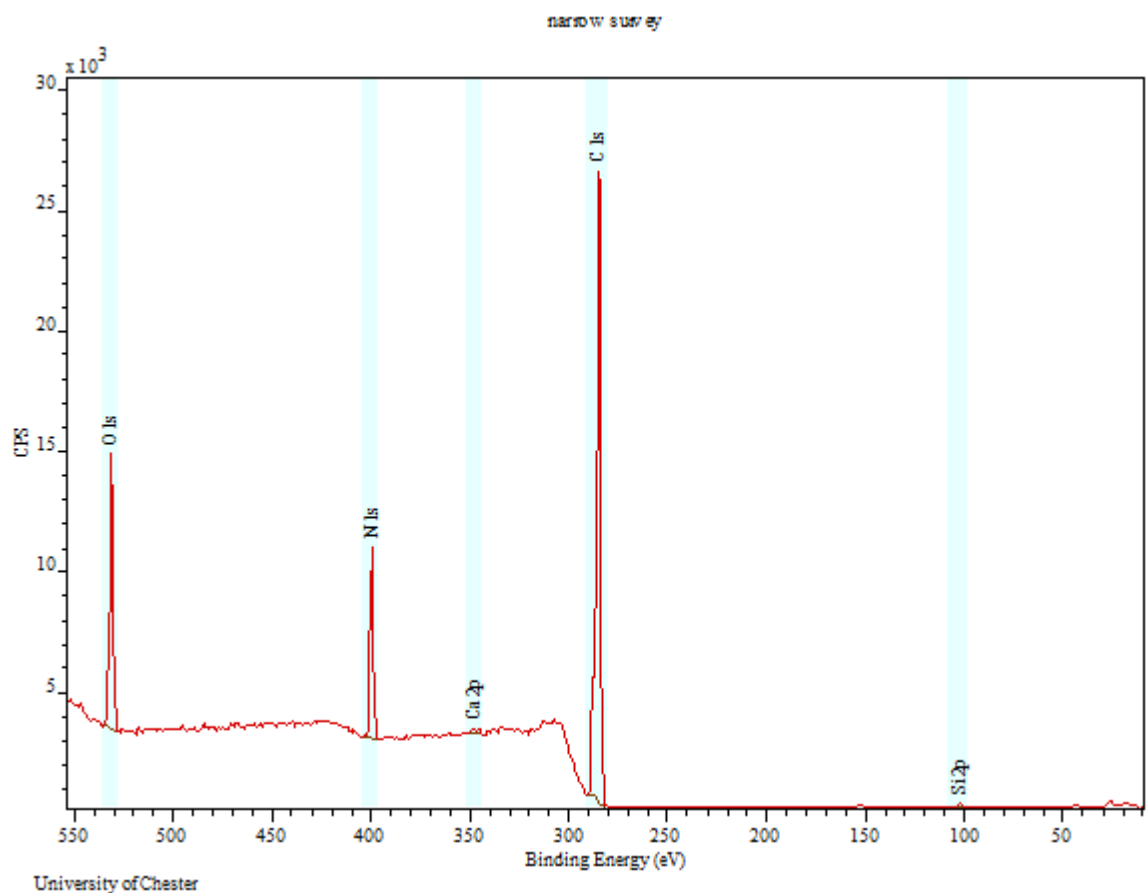


Table 21: A mass spectrograph showing the main elements present in Nylon sample M1, highlighting the main elements present.

Name	Conc (%)
C 1s	77.68
O 1s	11.14
N 1s	10.77
Si 2p	0.3
Ca 2p	0.12

Table 22: A tabularised list of the main elements present in M1-N

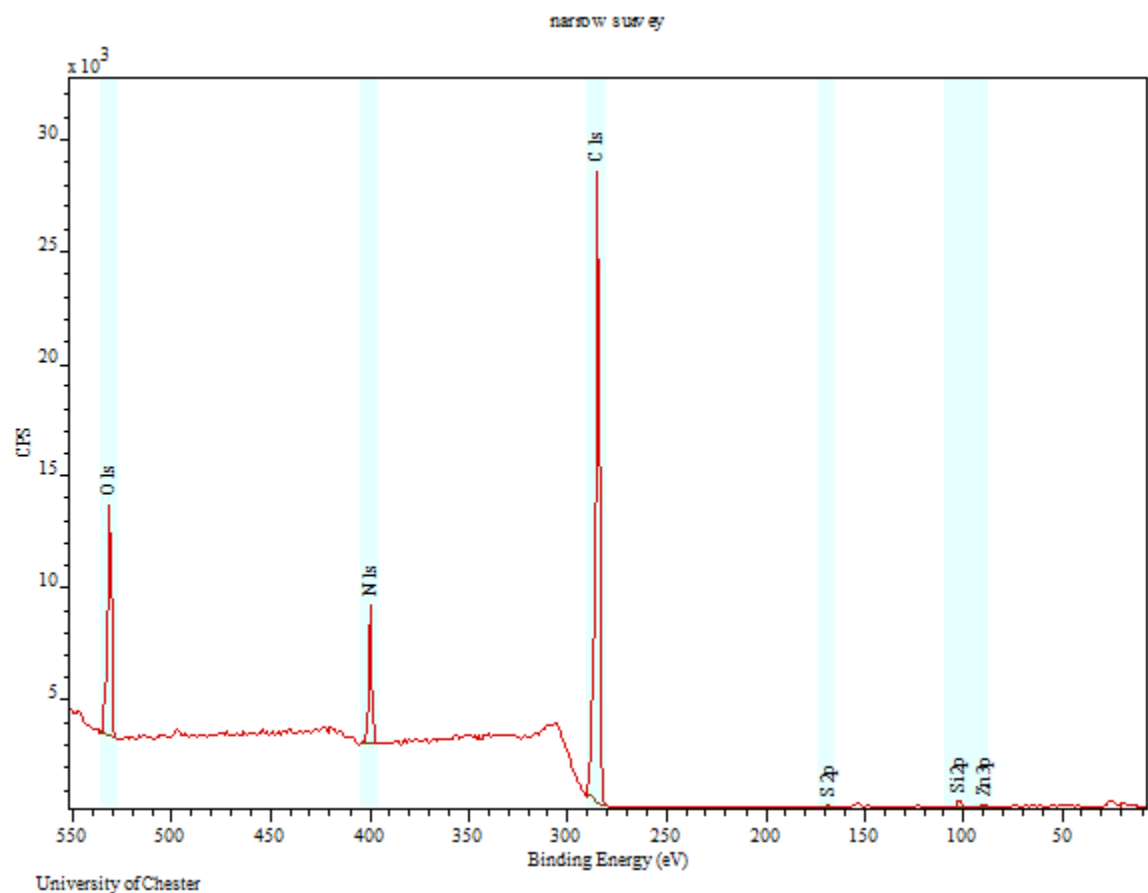


Table 23: A graph showing the elemental content of M2-N, highlighting the most abundant elements.

Name	Conc (%)
C 1s	79.41
O 1s	11.42
N 1s	8.37
Si 2p	0.7
S 2p	0.06
Zn 3p	0.04

Table 24: The key elements present in sample M2-N by percentage.

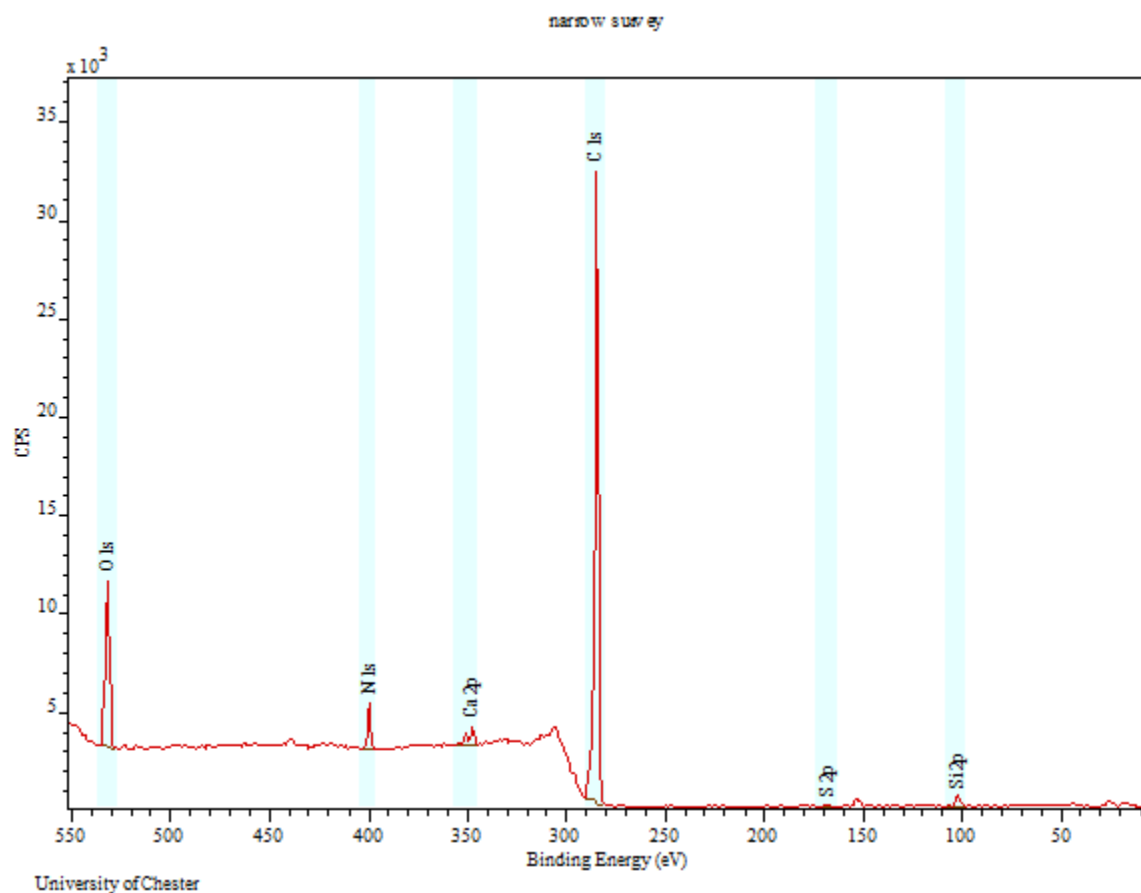


Table 25: Graph showing the elemental content of sample M3-N, highlighting the most abundant elements found.

Name	Conc (%)
C 1s	79.55
O 1s	11.03
N 1s	8.86
Si 2p	0.51
S 2p	0.05

Table 26: List of the most abundant elements in M3-N by percentage concentration

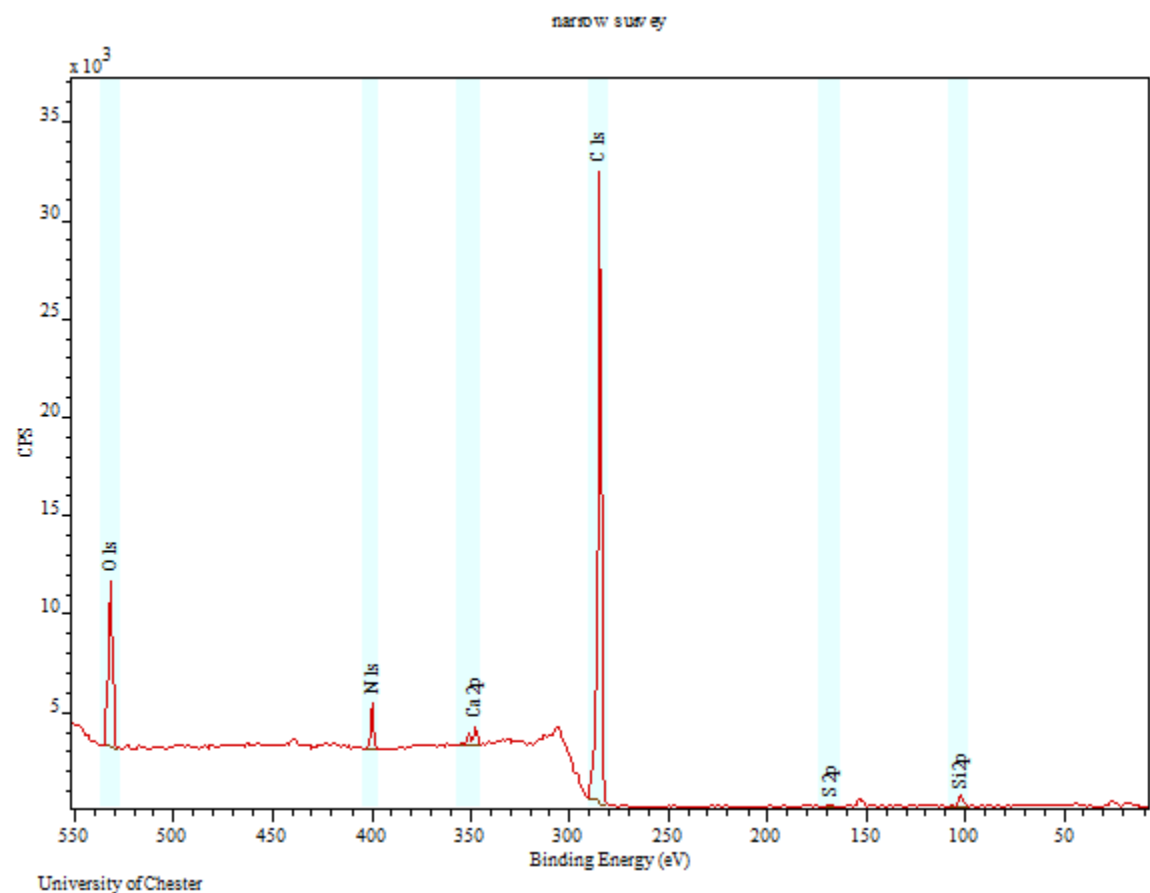


Table 27: Mass spectrograph showing the chemical profile for M4-N, highlighting the most common elements found

Name	Conc (%)
C 1s	83.52
O 1s	10.9
N 1s	3.32
Si 2p	1.48
Ca 2p	0.68
S 2p	0.1

Table 28: The most common elements found in M4-N as shown by percentage.

1. Material * Time

Measure: MEASURE_1

Material	Time	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
PEEK	1	168.508	5.042	157.992	179.025
	2	174.391	4.778	164.424	184.358
	3	187.247	4.978	176.864	197.631
Nylon 6'6	1	131.221	5.042	120.704	141.737
	2	153.221	4.778	143.254	163.188
	3	151.999	4.978	141.616	162.383

Table 29: A pairwise comparison of the mean absorbance values recorded during cytotoxicity testing for both materials used in this study PEEK & Nylon 6'6